A study of *Scutus breviculus* (Gastropoda: Prosobranchia) in marine and estuarine environments, with special reference to blood composition and nerve conduction.

A thesis presented for the degree of Doctor of Philosophy in Zoology in the University of Canterbury, Christchurch, New Zealand.

by

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In 1958 Kinne wrote "What happens in an organism that is adapting to different salinities? Which structural or functional alterations can be observed? Are these alterations primarily expressed on an enzyme, protoplasmic, cellular, organ or nervous level? What is the physiological meaning of these alterations? We know almost nothing about these questions." During the ten years since Kinne wrote this, there have been many papers published on salinity adaptation, but the same questions that he asked can still be asked today.

It is known that most marine animals which invade estuaries are able to survive in a variable brackish-water environment, not because they can regulate the concentration of their blood independently of the environment, but because they can tolerate large changes in the concentrations of their body fluids. Estuarine animals usually remain almost isosmotic with their environment, and only become hyperosmotic in very dilute brackish water (e.g. see Neumann, 1960; Todd 1964a, 1964b). The tissues of estuarine animals are able to function in a wide range of blood concentration, but most marine forms are unable to maintain their normal vigour in salinities below about $30^{o}/_{oo}$ (Potts and Parry, 1964).

It has often been shown that the normal functioning of nervous tissues of molluscs is affected by the osmotic pressure and the concentrations of individual ions in the surrounding fluid. Much of the electrophysiological work in this field of research has been done using the

giant nerve fibres of the squid. However, nerve function in gastropods has been investigated by many workers (e.g. Ramsay, 1940; Hughes and Kerkut, 1956; Kerkut and Taylor, 1956; Tauc, 1958; Tauc and Gerschenfeld, 1960; Hagiwara, Kusano and Saito, 1961; Nisbet, 1961). These experiments have been made with animals from marine and terrestrial habitats. Several neurophysiological studies have been carried out on fresh water lamellibranch species (e.g. Salánki, Lábos and Nán, 1964). It was thought that it would be of interest to study an animal which was subjected to variable salinity in its natural environment, to see how this variation in the concentration of ions in the external medium might affect the concentration of the blood and, if the concentration of the blood was found to vary, to see if this affected the functioning of the nervous tissue. Kinne (1964) stressed that a detailed evaluation of the effects of salinity as an ecological factor required information obtained both in nature and under specific conditions in the laboratory, and that the information obtained in nature should include the fluctuation patterns of salinity. In 1967 he wrote, "In estuarine organisms acclimation to environmental variations appears to be of greater importance than acclimations to extremely low or high intensities. Most of the experimental work on physiological aspects of non-genetic adaptation, however, has been carried out by exposing the test organisms to constant low, normal, or high factor intensities; information on acclimation to fluctuating conditions is lacking.... There is urgent need for new studies on non-genetic adaptation in est-

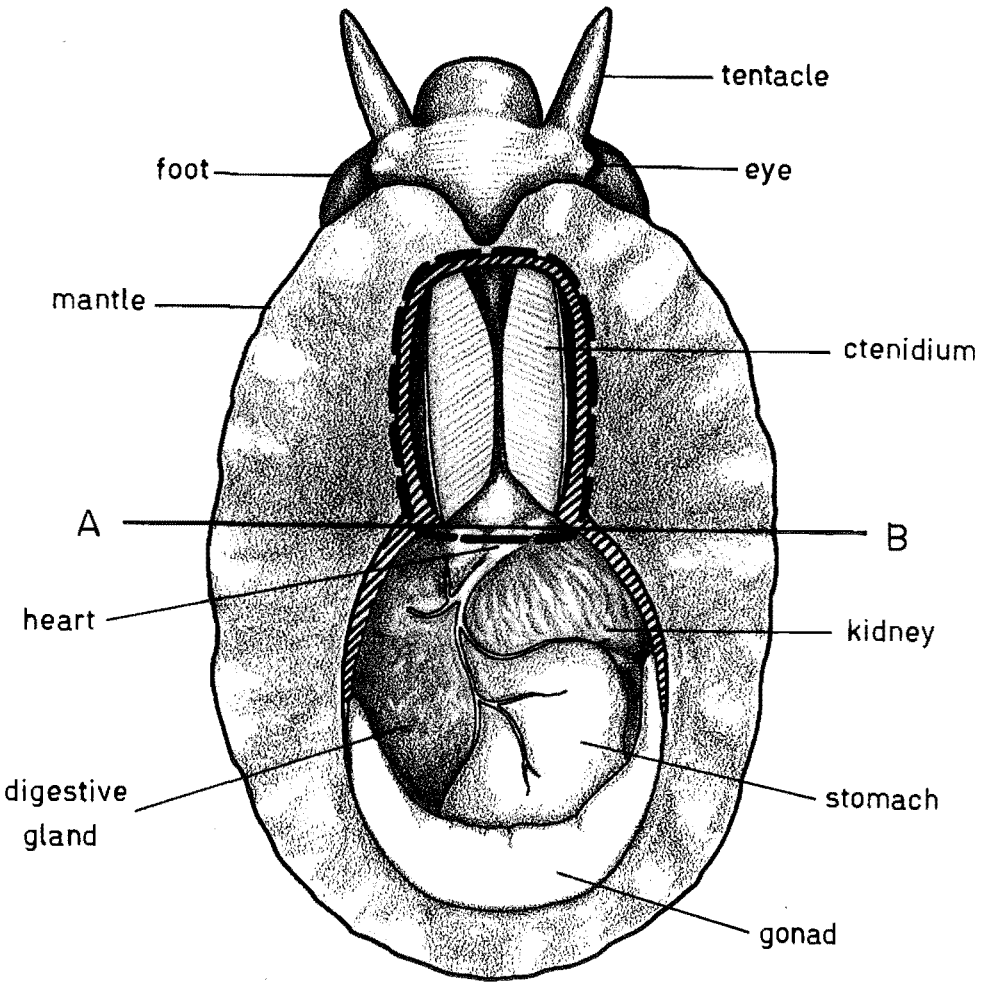

Figure 1: View of Scutus specimen dissected from the dorsal side. (adult, approx. natural size.) A-B is the plane of the section shown in Figure 8.
▬▬ Extent of shell.

uarine organisms, performed under conditions simulating nature."

The Fissurellid gastropod, Scutus breviculus* (Fig. 1) was chosen as the species which would be used in this study, planned to investigate some of the effects of variable salinity on the physiology of the animal. S. breviculus is normally a marine species, but it is also common in an estuarine environment near Christchurch. It was decided to subject animals to various dilutions of sea water (such as might be encountered in the natural environment, as well as conditions more severe than those) and to measure the osmotic pressure (estimated by the depression of the freezing point), and the sodium, potassium, calcium and magnesium concentrations of the blood of the animals when they were placed in the experimental media. These cations were chosen for analysis as, in addition to being the common cations in blood and sea water, they are all known to be important in conduction and/or transmission of nervous impulses. After analysis of the blood had been completed, artificial physiological solutions, with ionic composition close to that which had been found for the blood of animals in different experimental media, were prepared. These solutions were used to bathe the nerves during experiments examining the effects of the composition of the medium on conduction in the nerves.

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* See Appendix I for full classification.

SECTION 1

SCUTUS AND ITS HABITAT

1.1 PREVIOUS WORK ON SCUTUS

There has been very little published about the genus Scutus, since de Montfort classified it in 1810. Near the end of the nineteenth century Boutan (1884, 1890a, 1890b, 1891a, 1891b) and Bouvier (1887) carried out some anatomical studies on specimens of Parmophorus australis (=Scutus australis), which had been collected from Port Jackson, Australia. (This was the species which is now called S. antipodes and which is distributed throughout southern Australia.) Most of the work published was on the nervous system (see Section 3), but that of Boutan also included a short paper (1891a) on the mantle and shell, and one (1891b) on the larval form. Pelseneer (1890) wrote a short article on the mantle of Scutus and compared its form and position with that of other molluscs.

A fairly comprehensive anatomical study of Parmophorus intermedius (=Scutus breviculus) was made in Germany by Tobler in 1902, but not much of this is relevant to the present study since he included very little information on the blood and nervous systems.

In New Zealand two theses have been presented on S. breviculus. Webber (1931) wrote a thesis on its anatomy, but no copy of this is available. Milligan (1955) also wrote a thesis about this species, and it included short sections on feeding, growth, reproduction and the sex ratio, ctenidia, pallial ciliary currents, and the shell. He also studied the digestive tract and

described its gross and microanatomy. Much of Milligan's work on the anatomy was a repetition of that which Tobler had done earlier. Milligan did state that some of the details of the anatomy described by Tobler for '*Parmophorus*' were very similar to those which he found in *Scutus*.

Morton (1952) has studied the role of the crystalline style in *S. breviculus* and Owen (1958) has made observations on the stomach and the digestive gland.

1.2 DISTRIBUTION OF SCUTUS

Simroth (1896-1907) reported that fourteen species of *Scutus* were recorded from Tertiary deposits of the Paris Basin, and that the genus was now restricted to the Madeira Islands and the Indo-Pacific region. Several species have been described for the Indo-Pacific region, but the nomenclature of these species has not remained stable; the distribution of species today appears to be

S. anatinus - Australia (Cotton and Godfrey, 1938)
Philippines (Faustino, 1928)
S. antipodes - Australia (Allan, 1950)
S. astrolabensis - Australia (Allan)
S. breviculus - New Zealand (Powell, 1957)
S. intermedius - Philippines (Faustino)
S. unguis (=*S. granulatus*) - Australia (Allan)
New Caledonia (Risbec, 1937)
Philippines (Faustino)
S. virgo - Japan (Habe, 1951)

Scutus breviculus is distributed throughout the Auporian, Cookian and Fosterian marine provinces of New

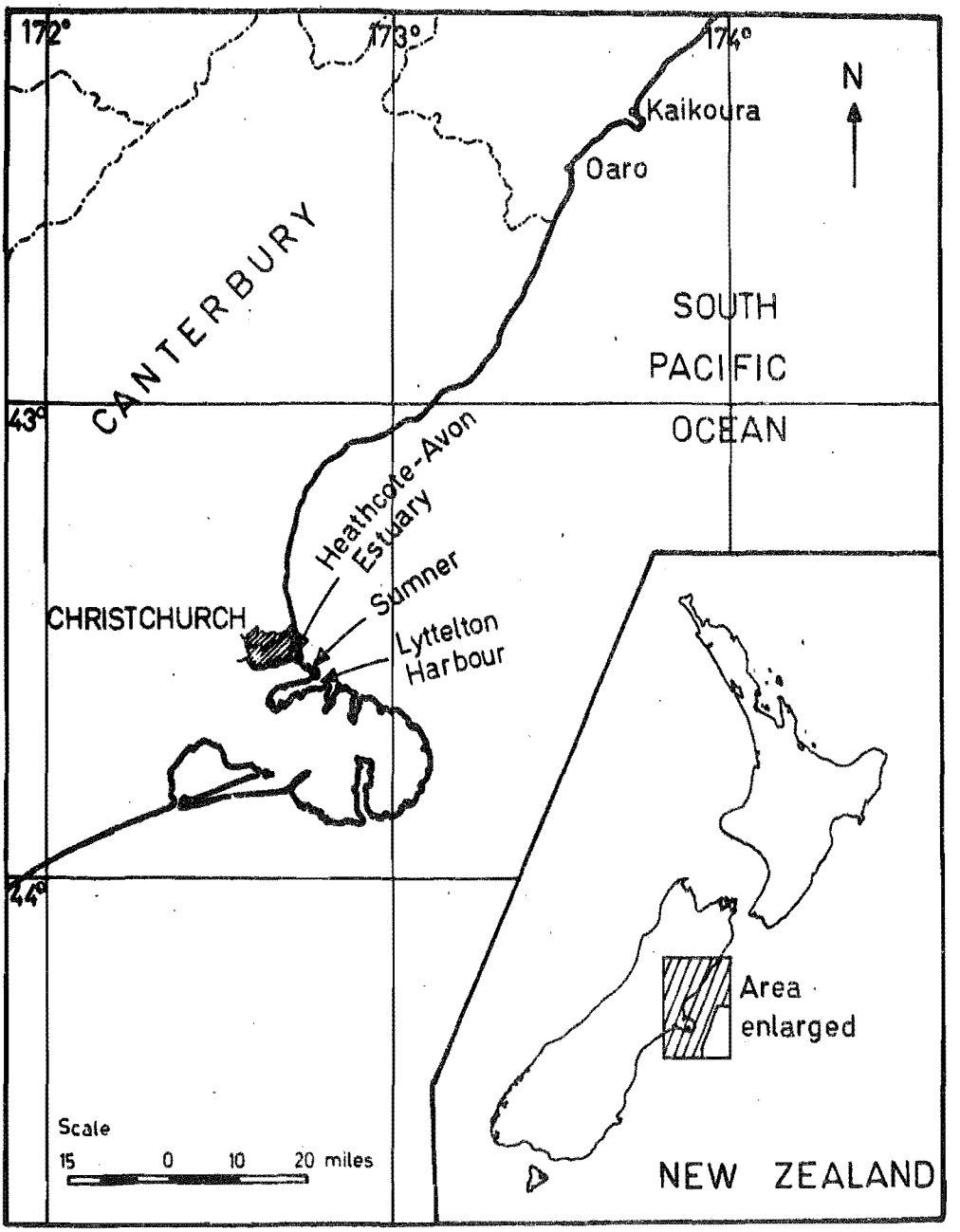

Figure 2: Map to show areas from which Scutus was collected

Zealand. It has not been recorded on the Chatham, Antipodes, Bounty, Auckland, Campbell, Macquarie or Kerguelen Islands, or any other islands of the Southern Ocean outside the New Zealand faunal region (Powell).

1.3 HABITAT OF SCUTUS*

Scutus is usually found in a marine habitat, under rocks or in crevices, at or below low water. A specimen has been reported at Kaikoura (Fig. 2) at a depth of about 60 feet (G. Poore, pers. comm.). However, near Christchurch it is abundant in some areas of the Heathcote-Avon Estuary.

The densest populations are found in regions where Ulva lactuca is plentiful. Milligan (1955) found that Ulva formed the bulk of the material ingested by Scutus in its natural habitat. Dense growth of Ulva is found in areas where sewage effluents are discharged into the water. In a Royal Commission Report (1911), presenting the results of investigations into the excessive growths of green sea-weeds in sewage-polluted waters, it was reported that Ulva could rapidly absorb nitrogen in the form of"saline ammonia". It was also noted that, while Ulva could grow freely without sewage pollution, the addition of nitrogen in a form which might be supplied

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* Throughout the rest of this thesis, wherever the generic name Scutus is used without a specific name it refers to the species Scutus breviculus. This was the only species on which experiments were carried out in this work. However, in places where a comparison of different species of Scutus is being made (for example, in the section on the anatomy of the nervous system), to avoid confusion, specific names are included.

by sewage was apt to produce a rapid increase in the weed. Hogan and Wilkinson (1959) considered the Heathcote-Avon Estuary to be virtually an oxidation pond for the treatment of polluted waters from the Heathcote River and various sewage effluents. They stated that such conditions would naturally encourage the growth of Ulva. Bruce (1952) also discussed the relationship between growth of Ulva and sewage pollution. Milligan observed that Scutus was found sparsely distributed except in situations where Ulva was plentiful. He found populations of greatest density at the culverts under the McCormacks Bay causeway (in the Heathcote-Avon Estuary, see Fig. 3) and at Scarborough, at the eastern end of Sumner Bay (Fig. 3) in the vicinity of an outflow of sewage. Scutus is still present in large numbers at these locations. He listed Lyttelton Harbour (Fig. 2) in the vicinity of Battery Point Military Camp, as another area where Scutus was common. This part of the shore-line of Lyttelton Harbour has since been changed by the construction of Cashin Quay.

The only other area which was found, during this study, to have a dense population of Scutus, was a rocky platform at Oaro, just south of the Kaikoura Peninsula (Fig. 2). This shelf also has abundant Ulva growth and, in addition, the green alga Enteromorpha sp. is plentiful. At Oaro Haliotis is also found in large numbers; this was the only area in which both Scutus and Haliotis were found to be common.

It was noticeable that the animals found in the estuary tended to be smaller than those of the populations at Scarborough and Oaro. When Milligan collected a sample of 1110 animals from the estuary in 1955, the largest animal collected weighed only 196.7 g

and only 6.67% of the sample weighed more than 100 g. Only a small number of animals from the estuarine habitat were weighed during the present study, and they were all found to be within the size range found by Milligan in his sample. At Scarborough and Oaro animals with weights greater than 200 g are common; in the present study the largest animal recorded at Scarborough weighed 316 g and at Oaro, 285 g. Marine organisms have been shown to exhibit a reduction in final size in areas of their distribution where the salinity is significantly reduced (Kinne, 1964) and numerous examples have been reported, especially from the North Sea and the Baltic. However, the generally smaller size of Scutus specimens in the estuary may not be directly related to the reduced salinity to which animals in that environment are subjected, but may rather be a reflection of the availability of food. There is also the fact that the estuarine habitat is more easily accessible to collectors than are Scarborough or Oaro; therefore, animals are more frequently removed from that area than from the two marine habitats, the larger animals being collected in preference to smaller ones.

1.4 ESTUARIES IN GENERAL

1.4.1 The estuarine environment

An estuary is defined (Cameron and Pritchard, 1963) as a semi-enclosed coastal body of water having a free connection with the open sea, and within which the sea water is measurably diluted with fresh water deriving from land drainage, i.e. the water is brackish. (According to the 'Venice System', brackish waters are defined as mixohaline waters of between 30 and 0.5‰ salinity

(Potts and Parry, 1964)). An estuary is usually small and shallow and is subjected to fluctuations of salinity caused by flooding and evaporation. Therefore, as differential precipitation of salts occurs during evaporation, the proportion of salts tends to be different from that found in normal sea water (Beadle, 1943). Howes (1939) found a significant increase in sulphate at the expense of chloride, and Emery, Stevenson & Hedgpeth (1957) stated that water in estuaries usually had a higher ratio of carbonate and sulphate to chloride, and of calcium to sodium, than did sea water.

In those estuaries which Day (1951) categorized as exhibiting a vertical salinity gradient, the incoming salt water does not mix completely with the river water, which is lighter and floats on the surface, so that salinity increases with depth. Day considered that, in the absence of turbulent currents, the sea water started to flow in along the bottom as soon as the tide began to rise, while the lighter river water was still flowing out at the surface. Panikkar (1950) noted that high salinities often prevailed in the silt-laden region of the bottom, while Milne (1940) and Topping & Fuller (1942) observed that salinity was higher in the substratum than in overlying water.

Variable salinity is not the only factor which might limit the invasion of estuaries by some marine forms. In fact, Allee, Park, Emerson, Park & Schmidt (1949) concluded that salinity did not ordinarily function as the limiting factor in the distribution of marine organisms. They pointed out that food supply,

pH and temperature might be the limiting factors in preventing extensions of organisms into salinity ranges which the organisms might otherwise tolerate. Pilgrim (1953) obtained evidence that adult animals of three molluscan species could survive quite wide fluctuations of salinity of their environmental media, and stated that restriction of their ecological distribution in respect to salinity seemed to be related to some other factor(s), for example, less tolerant stages in the life histories. In estuaries in temperate regions the range of temperature, both diurnally and seasonally, is much wider than in the sea. Sumner, Louderback, Schmitt & Johnston (1914) noted that at the entrance of an estuary the temperature is nearly the same as the open sea; upstream, however, the difference usually increases rapidly. In addition to horizontal temperature variation, considerable vertical variation may be produced, for example by warmer or colder river water flowing out over the more dense brackish water. Estuarine animals are also subjected to more variable oxygen tensions and water currents than are marine animals, and to much suspended matter in the water and, in many cases, to sustained precipitation of silt (Hunter, 1964).

1.4.2 Fauna of estuaries

Kinne (1967) listed four physiological mechanisms which may be possessed by estuarine organisms to assist them in compensating for the ill effects of the salinities experienced in their environment.

1) Escape, which is restricted to vagile organisms.
2) Reduction of contact by
   (a) producing slime, mucus or some other protective

substance to cover the body surface,

(b) retreating into holes or burrows,

(c) contracting muscles to reduce the surface to volume ratio of sensitive organs or of the whole body,

(d) withdrawing sensitive body parts, and

(e) closing shells or comparable structures. This mechanism is used by many marine molluscs which have invaded estuaries, e.g. Mytilus, Patella (Milne, 1940). They exclude the brackish water during that part of the tidal cycle when it is most dilute; however, the shell of Scutus is much reduced, so that the ctenidia and most other parts of the permeable body surface are exposed to the external medium at all times.

3) Regulation - ion regulation, volume regulation and osmoregulation.

4) Acclimation, a phenomenon by which organisms adjust to alterations in the intensity patterns of variation in their environment.

As has been already noted, the majority of marine molluscs are poikilosmotic, although van Weel (1957) considered that weak osmoregulation was more widespread in molluscs than had been thought to be. Potts (1954a), from energy considerations, came to the conclusion that the most important means whereby a marine animal entering brackish water could reduce the strain upon its osmoregulatory mechanisms was by reducing the concentration of its blood. Bassindale (1943) pointed out that it was not just the degree of dilution that was the

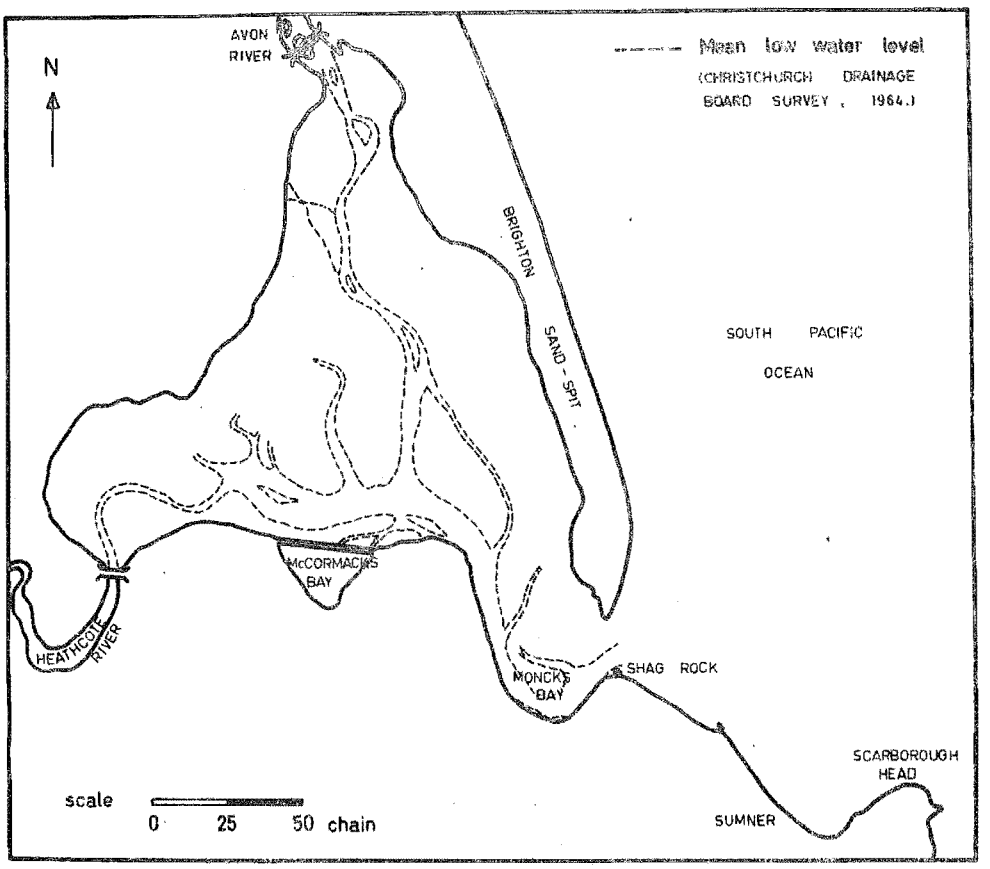

Figure 3: Map of the Estuary of the Heathcote and Avon Rivers, and Sumner Bay

limiting factor in the distribution of animals, but that the rate of change of salinity was also significant. Many animals could adapt themselves to large changes if they occurred slowly enough.

The number of marine species in an estuary declines rapidly with salinity (see Dahl, 1956). Hedgpeth (1951) noted that, in the lower part of an estuary, the fauna was predominantly marine and that it consisted, for the most part, of species occurring in the neighbouring seas. Of these, the littoral species, already accustomed to daily changes in their environment, were the most obvious. Percival (1929), after dealing with the components of the population of the Tamar Estuary and describing the penetration of different systematic groups, gave a diagram of distribution in relation to salinity. From this it would seem that most of the marine species disappeared when the salinity fell below about 30-25$^{o}/_{oo}$. From observations made by Nicol (1935), it is evident that there is a big drop in the number of marine species between salinities of 35 and 25$^{o}/_{oo}$. In estuaries with a vertical salinity gradient, typically marine species may be found in the bottom parts in some locations of the estuary, while being excluded from the surface layers where the water is more dilute (Panikkar, 1950).

1.5 THE HEATHCOTE-AVON ESTUARY

1.5.1 General description

The location of the estuary of the Heathcote and Avon Rivers is shown in Figure 2. It is a bar-built estuary (according to the classification of Pritchard (1967)), and it covers a large expanse of tidal mud

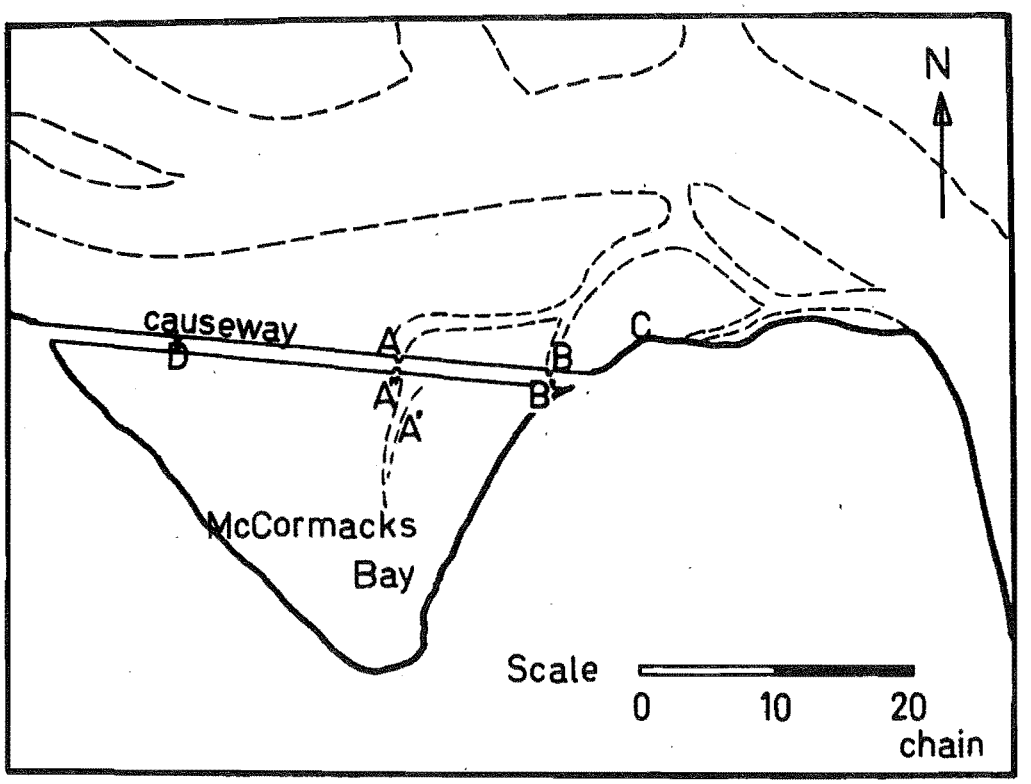

Figure 4: Map to show sampling stations A-C at McCormacks Bay, where salinity measurements were made. (Location D was not used as a sampling station in the present study, but Fearon (1962) made salinity determinations on water collected at this station. His results are included in Table 2.)

flats, taking the form of a roughly equilateral triangle, with sides about two miles long (Fig. 3). The Heathcote River enters the estuary in the south-western corner, the Avon River in the north and the outlet to the sea is in the south-east. On its eastern side, a bar (the Brighton sand-spit) separates the estuary from the sea. At McCormacks Bay a causeway cuts off 115 acres from the main body of water.

Pritchard (1967) notes that, in bar-built estuaries, tidal action is considerably reduced. In the Heathcote-Avon Estuary, Thompson (1929-30) found tides varied from four to six feet in height. (At Lyttelton tides of up to eight feet occur (New Zealand Tide Tables, 1966-1968)). He observed that the wind had a pronounced effect on the height of tides, an easterly wind, (either a north-easterly or a south-easterly) tending to bring the tide in a little earlier, raising it to a greater height, and keeping it in a little later than did a westerly wind. As the tide ebbs, the large volume of water in McCormacks Bay is channelled out through three narrow culverts under the causeway, but the bay never becomes completely empty. It is under rocks on the seaward side of one of these culverts (Sampling station B, Fig. 4) that Scutus is most abundant in the estuary. Scutus in this location has a continuous supply of well-aerated water.

1.5.2 Salinity changes

Methods

Estuarine water samples were collected from six sampling stations (Fig. 4, A-C) in areas where Scutus was present. At sampling stations A and B (on the

TABLE 1

Maximum and minimum salinities measured at six estuarine sampling stations. (For stations A and B the salinities of both the surface and bottom water are shown.)

Sampling Station	Salinities measured		
	Maximum	Minimum	
	S°/oo	S°/oo	% of maximum
A (surface water)	33.80	26.58	78.64
(bottom ")	33.74	29.01	85.98
A') adjacent to	33.86	33.03	97.55
A") Scutus	33.68	30.34	90.08
B (surface water)	33.61	25.04	74.50
(bottom ")	33.82	30.00	88.70
B') adjacent to	33.77	33.23	98.40
C) Scutus	33.45	31.30	93.57

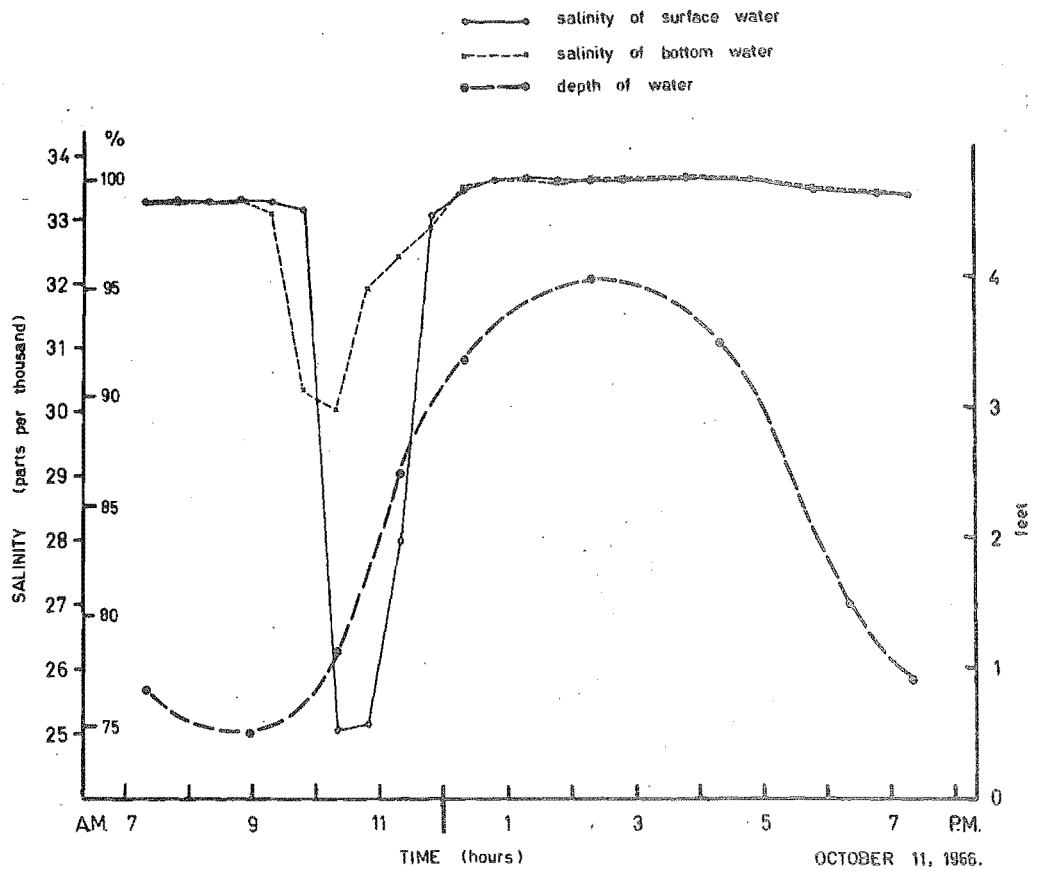

Figure 5: Changes in salinity of the surface and bottom water, and depth of water, during a tidal cycle at sampling station B, McCormacks Bay.

seaward side of two of the culverts under the causeway) both surface and bottom water samples were collected at intervals throughout a complete tidal cycle. Surface water samples were collected in polystyrene containers which were capped tightly immediately after collection of the samples. Bottom water samples, in the immediate environment of Scutus, were collected with a van Dorn-type water sampling bottle and then transferred to capped polystyrene containers. All samples collected at other sampling stations (A', A", B', C) were taken immediately adjacent to Scutus specimens. Samples were collected at various times throughout the year.

The majority of salinity measurements were made using an inductively coupled salinometer, manufactured by Auto-Lab. Industries. According to the specifications of the manufacturers, this instrument has an accuracy of approximately 0.003 parts per thousand for salinity measurements.

When the salinometer was not available, salinities were determined by the Mohr titration (Strickland and Parsons, 1960). This method is correct to about 0.1 parts per thousand.

Results

Table 1 shows the maximum and minimum salinities recorded at the six estuarine sampling stations during this study. The highest salinities were recorded during the summer months.

Figure 5 shows the salinities at station B (surface and bottom water) throughout a complete tidal cycle. Figure 6 illustrates the correlation between the

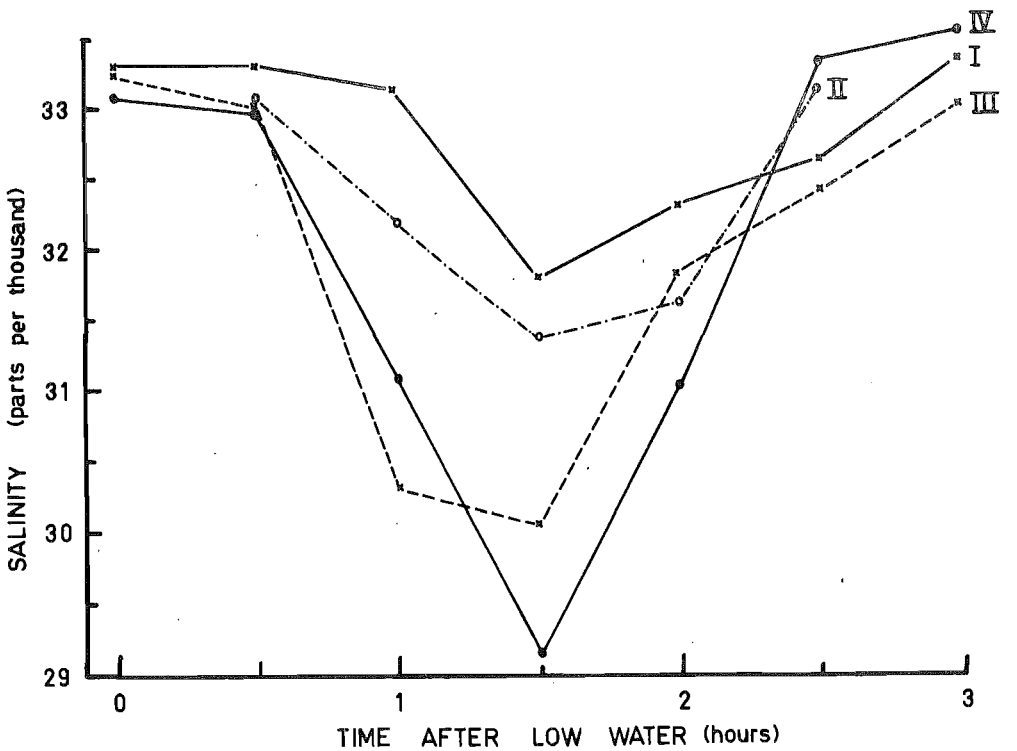

Figure 6: Effect of prevailing wind on the degree of dilution of the bottom water on the seaward side of the culverts at McCormacks Bay.

I	Sampling station	B:	October, southwesterly wind
II	"	"	B:	March, light easterly breeze
III	"	"	B:	October, strong easterly
IV	"	"	A:	October, strong easterly

TABLE 2

Minimum and maximum salinities, and range of salinity, within a tidal cycle at culverts under the causeway at McCormacks Bay.

Culvert (see Fig.4)	Month	Maximum $S^{o}/_{oo}$	Minimum		Range $S^{o}/_{oo}$	Surface (s) or bottom (b) water	Reference
			$S^{o}/_{oo}$	% of max^{m}			
A	Jan.	33.6	21.4	63.7	12.2	*	Milligan (1955)
A	June	33.8	28.5	84.3	5.3	*	"
D	April	30.5	25.9	84.9	4.6	*	Fearon (1962)
A	May	33.90	25.40	74.9	8.50	s	Dix (unpublished)
B	March	33.50	31.30	93.4	2.20	b	Present study
A	October	33.59 33.61	26.58 29.01	79.2 86.3	7.01 4.60	s b	
B	October	33.60 33.61	25.04 30.00	74.5 89.2	8.56 3.61	s b	
B	October	33.45 33.50	28.05 31.82	83.8 95.0	5.40 1.72	s b	

* Milligan & Fearon did not record how samples were collected.

prevailing wind and the degree of dilution of water at the culverts, at that time of the tidal cycle when the salinity is noticeably reduced.

The sea water at Scarborough is noticeably diluted by the outflowing estuarine water. A sample taken from Scarborough soon after low tide had a salinity of $32.9^{o}/_{oo}$, compared with the lowest measured salinity of water collected at Lyttelton of $33.5^{o}/_{oo}$.

Results from other workers

Several other workers have studied the salinity changes occurring at various sampling stations, ranging from near Shag Rock at the outlet of the estuary, to the regions where the rivers enter the tidal flats. A few have taken samples from near McCormacks Bay causeway, and other rocky parts of the shoreline where Scutus is found between Moncks Bay and McCormacks Bay. Salinity values recorded by Milligan (1955), Fearon (1962), and Dix (unpublished) at culverts under the McCormacks Bay causeway are given, along with some data obtained in the present study, in Table 2. Linzey (1944) observed that the salinity of the permanently filled portion of McCormacks Bay showed an appreciable rise owing to evaporation during the summer months, but he gave no salinity values to substantiate his statement.

Discussion

Salinities observed at sampling stations A and B on the seaward side of the culverts under the causeway show a sharp decrease about half an hour after low water and, very soon after, a rapid increase to a level which is maintained fairly constant throughout the rest of the tidal cycle. The sharp decrease is brought

about by estuarine water returning with the incoming tide. The water leaving the estuary soon after high water contains less river water than that leaving just before low water. It is this latter water (with the high proportion of river water) that returns with the incoming tide. Bruce (1952) calculated that 41% on one occasion, and 32% on another, of the river water that left the estuary returned on the following tide. The returning estuarine water of low salinity is forced back over the tidal flats as the tide begins to flow. As water of higher salinity, from the open sea, mixes with the estuarine water later in the tidal cycle, the salinity of the estuarine water rises. Estcourt (1962) noted that more salt water entered the estuary at spring tide than at neaps. Although there is a similar pattern of salinity change, with a drop in salinity for a short time after the tide begins to flow, during both spring and neap tides at different times of the year the range of salinities within different tidal cycles shows considerable variation. This variation appears to at least some degree to be dependent on the prevailing wind (Fig. 6). Lowest salinities were recorded when there was a strong easterly wind. This indicates that a strong easterly wind forces the returning estuarine water, with a high proportion of river water, further back up the channels of the estuary with the incoming tide. This is not inconsistent with the observations which Thompson (1929-30) made concerning the relationship between the direction of the prevailing wind and the tides in the Heathcote-Avon Estuary.

The decrease in salinity was much more marked in the surface waters than in the bottom water in the region of the culverts at McCormacks Bay, i.e. there

was a noticeable vertical salinity gradient. (Therefore, judging from the low salinities which they recorded, Milligan and Fearon would appear to have taken their samples from the surface water, which would have no direct effect on the animals living under rocks at the bottom in these areas.) Linzey (1944) studied salinities in the Heathcote-Avon Estuary and in discussing the phenomenon of layering (vertical salinity gradient) wrote "Evidence pointed to a fairly extensive occurrence of this interesting phenomenon, but no quantitative estimation was made."

1.5.3 Temperature

As temperature changes have been reported to have an effect on the ability of an animal to osmoregulate (see, e.g., Gunter, 1957; Verwey, 1957; Kinne, 1964), it was decided to take temperature measurements in the McCormacks Bay area, in order to obtain an indication of the extremes to which Scutus was likely to be subjected in its natural environment.

The lowest temperature recorded was 6.5°C and the highest 24.5°C. The extreme temperatures were measured at about the time of low water, the minimum was taken at 7.30 a.m. and the maximum at 3 p.m. Johns (1960) has reported a minimum temperature of 5°C, and Milligan a maximum of 25°C at the end of the outflow from McCormacks Bay during the summer. Milligan found that below about 3.5°C Scutus could not maintain its hold on a vertical glass surface.

In connection with the foregoing it was decided to conduct some experiments in a controlled temperature room at 7°C (close to the minimum found in the estuary); another set of experiments was conducted at room temp-

erature, in which aquarium temperatures varied between about 17° and 19°C. The results are described in section 2.3.5.

SECTION 2

WEIGHT CHANGES AND BLOOD COMPOSITION

2.1 INTRODUCTION

Fredericq (1885, 1901), Bottazzi (1897), Quinton (1900), Macallum (1903) and Dakin (1908) were among the first workers to study the relationship between the composition of the blood of marine animals and that of the external medium. As early as 1885, Fredericq observed "Le sang des Mollusques d'eau douce est pauvre en sels, tandis que celui des Mollusques marins a exactement le même goût que l'eau de mer dans laquelle ils vivent". Quinton and Bottazzi both noted that the salt concentration of the blood of marine invertebrates was equal to, or very close to, that of the sea water which surrounded them. Dakin observed that the blood and coelomic fluids of both aquatic vertebrates and invertebrates were in equilibrium with the external media, though perhaps differing very much from it in the concentrations of individual ions; any changes in the constitution of the external medium brought about a gradual change in the blood, to a greater or lesser degree in different groups, until a new equilibrium was set up. Many of the early workers were of the opinion that the surfaces of aquatic animals were permeable to water, but not to salts and other substances present in the blood and in sea water; i.e. they were "semipermeable", in strict chemical terminology. This view was based on the results of experiments which showed that when an animal was placed in diluted sea water there was a decrease in

the osmotic pressure of the blood and, at the same time, an increase in the body weight of the animal. The conclusion drawn was that the equalization of the internal with the external osmotic pressure was brought about by the diffusion of water alone.

However, Quinton (1900) obtained evidence that the body walls of Aplysia, Sipunculus and Carcinus were permeable to chloride and phosphate ions, as well as to water. Macallum (1903), after conducting experiments on Aurelia flavidula, came to the conclusion that, for this species, the cells lining the gastro-vascular channels, and perhaps to some extent those covering the organisms, exerted a selective influence in absorbing the salts of sea water. Using Aplysia as an experimental animal, Bethe (1930) obtained evidence of permeability of the body wall to several cations. He made calcium-free artificial sea water and found that when animals were placed in this medium there was a decrease in the amount of calcium in the blood; in these conditions the blood calcium concentration of Aplysia decreased to one third of its original value within five hours. He repeated this experiment with potassium- and magnesium-free sea water, and then with these ions augmented. In each case he found that there was a corresponding movement down the concentration gradient, out of or into the animal. Bethe also conducted experiments in which other species of soft-skinned marine animals (Sipunculus, Holothuria and various medusae and pelagic molluscs) were transferred to sea water diluted with isotonic non-electrolyte solutions. From the results of these experiments he concluded that the surfaces of all the marine invertebrates on which he had experimented were permeable to water and also to the ions of salts which were present in their blood and in sea water. He

considered that the forces which tended to bring the salt content of the blood into equilibrium with the surrounding sea water were just as great as the forces which strove to prevent the osmotic differences. The skin of those animals, except in cases where special modifications had arisen, served only as a protective barrier preventing the loss of body colloids.

Several workers have investigated osmoregulation of prosobranch gastropods in dilute and concentrated media. Garrey (1905) reported on several species, all of which were isosmotic in 100% sea water. The prosobranch Busycon canaliculatum tested in 50% sea water (Δ_e = 1.02) showed a decrease in the osmotic concentration of the blood (to Δ_i = 1.07, within 30 hours). Todd (1964a), Avens (1965) and Avens & Sleigh (1965) have studied osmotic pressure changes in the blood of the brackish water prosobranch Hydrobia ulvae, in which body fluids were found to be isosmotic with the medium over a wide range of sea water concentrations. Effects of changes of the salt content of the medium on the osmotic pressure of the body fluids of the fresh water species Viviparus fasciatus and Theodoxus fluviatilis were studied by Obuchowicz (1958) and Neumann (1960), respectively. Little (1965), in addition to studying the osmotic pressure changes in the blood of Viviparus viviparus, made determinations of various cations in blood samples. Segal and Dehnel (1962) found that the intertidal prosobranch Acmaea limatula did not osmoregulate over the range of salinities 25% to 150% sea water; using an equation formulated by Gross (1954), they were able to calculate 'mobile water' values which showed that the blood concentration changes in different salinities were affected by salt as well as water movement, particularly at the

lower external salinities. Todd (1964b) found that Littorina was essentially isosmotic in sea water of 150-50%, and was increasingly hyperosmotic in salinities of from 50-25% sea water. In the salinity range in which the animals were active (150-50%), the Littorinidae reached a new steady state of osmotic concentration within 24 hours after transfer to a lower salinity, without any marked change in volume, suggesting that the lower osmotic concentration involved a salt loss.

Robertson (1941) stated "The body walls of all marine invertebrates are permeable to sea water ions, and it is not surprising that alteration of the external medium causes corresponding changes in the internal medium and various physiological effects on these organisms and their tissues."

Robertson (1949, 1953, 1964) has investigated ionic regulation of many invertebrates, including two prosobranchs, Neptunea antiqua and Buccinum undatum. He determined concentrations of ions in the plasma, and in plasma which had been dialyzed against sea water and concluded that, even in 100% sea water, the differences between the concentrations of various ions in the body fluids and the concentrations of these ions in sea water were due to an active regulation, and not simply to a passive equilibrium between the body fluids and the surrounding medium. In gastropods, regulation was found to result chiefly in raised values of potassium and calcium, and lowered values of sulphate; sodium and chloride were virtually in equilibrium across the boundary membranes of the animals. Magnesium was found to remain at an equilibrium value in practically all the gastropods studied, but was slightly higher in the blood

of the opisthobranch Archidoris. The differences which remained after dialysis were so small compared with the differences between normal blood and sea water, that it was concluded that protein binding was insignificant as a regulating mechanism in maintaining ionic differences between blood and sea water. A study of ionic regulation in the shallow-water marine queen conch, Strombus gigas, was made by Little (1967).

This work by Robertson and Little on ionic regulation did not include analyses on animals which had been placed in dilute or concentrated media. When the experiments on ionic regulation in Scutus were carried out, there appeared to be no information available about rates of ionic concentration changes in the blood of marine prosobranch gastropods placed in dilute or concentrated media. However, recently, Webber and Dehnel (1968) have published a paper on ion balance in the prosobranch gastropod Acmaea scutum and, in the study reported in this paper, analyses of cations in the blood, urine and cellular spaces of animals placed in hyper- and hypotonic media were made at intervals between 1.5 hr and one week after the animals had been placed in the experimental salinity. Animals used for these experiments were collected both from marine and from estuarine habitats. The salinity range (6.4-25.5$^{o}/_{oo}$), to which the estuarine limpets were exposed in their natural environment, was much wider and lower than that (29.0-33.6$^{o}/_{oo}$) to which Scutus is subjected in the Heathcote-Avon Estuary. Webber and Dehnel found that, after a period of time in experimental salinities ranging from 50-125% sea water, the blood Na^{+}, Cl^{-} and Mg^{2+} concentrations in Acmaea were not significantly different from the concentrations of the respective ions in the experimental salinities. However, for estuarine animals (previously kept in 75-80% sea

water), in 75% and 50% sea water the concentration of Ca^{2+} in the blood was slightly higher than that in the medium. For marine limpets at all salinities and estuarine limpets in 125% sea water, there was no difference between blood and sea water Ca^{2+} values. The concentration of K^{+} in the blood of both estuarine and marine animals was always greater than that in the surrounding sea water; the equilibration time for the new K^{+} values to be reached varied considerably, being 3-48 hours for animals placed in 75% and 125% sea water, and 12-48 hours for those in 50% sea water. Curves of the total blood ion concentration also showed variability in the equilibration time of both estuarine and marine animals in the experimental media.

In this study experiments were planned to investigate osmotic pressure, and sodium, potassium, calcium and magnesium concentrations in the blood of Scutus, when subjected to

(a) salinity changes similar to those normally experienced by the animal during a tidal cycle at the culvert in the Heathcote-Avon Estuary, where Scutus is plentiful, and

(b) salinity changes which would be more severe than those experienced by animals in the natural environment. The three concentrations chosen for these experiments were 75%, 85% and 115% sea water. (75% sea water was approximately the minimum concentration measured in this study for the surface water at the culverts under the McCormacks Bay causeway (see Table 2, facing p. 15), and 85% sea water approximately the minimum concentration measured for the bottom water. 115% sea water was chosen as a

hypertonic medium; in this an animal would be subjected to a salinity change of the same order as the 85% sea water, hypotonic medium.)

During these experiments changes in wet tissue weight were also recorded.

2.2 METHODS

2.2.1 Treatment of animals before experiments

Male and female animals were collected from Scarborough, the Heathcote-Avon Estuary or Oaro, near Kaikoura. After being brought into the laboratory, they were placed in aquaria of sea water collected from Lyttelton, without food, for at least 48 hours before the commencement of any experiments. In the following experiments this sea water is referred to as 100% sea water; its concentration varied slightly from experiment to experiment (Table 3), as different containers of sea water were used.

2.2.2 Changing the concentration of sea water along a specified concentration gradient

All dilution and concentration experiments were carried out in an eleven litre aquarium, the water being continually aerated and mixed by air bubbled through a diffusion block. Never more than three animals were placed in the aquarium for any experiment.

In one group of experiments, changes in the medium concentration were regulated to imitate those found during a tidal cycle at the estuary where Scutus is found. In the laboratory, experimental media were diluted with deionised water and concentrated again with either boiled-down sea water, or an artificially prepared solution. To achieve this dilution (and concentration) along a predetermined concentration gradient, water (or

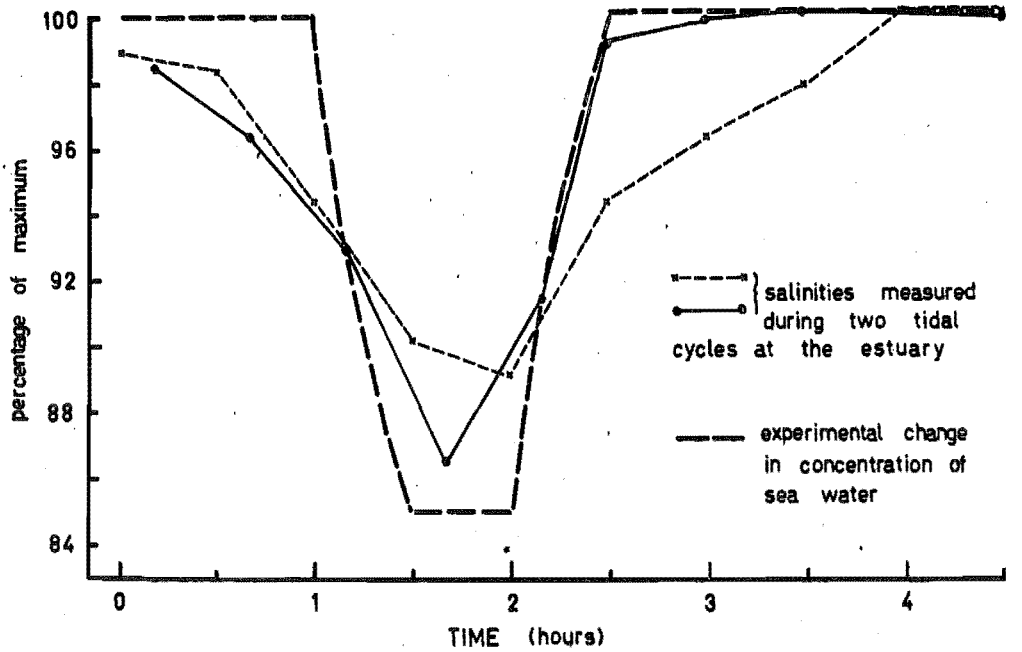

Figure 7: Dilution of sea water in the environment and that produced in the laboratory.

concentrated sea water) was run into the aquarium from a constant pressure bottle, the rate of flow being controlled by a needle valve. The solution overflowed from the aquarium at the same rate as the water (or concentrated sea water) was run in. (See Appendix II for detail of method). The curve of these salinity changes, which were carried out in the laboratory, is shown in Figure 7. Also plotted on this graph are two curves showing environmental salinity changes measured during two tidal cycles at the estuary.

2.2.3 Weighing procedure

Before animals were weighed, excess water was shaken from the pallial cavity, and surface water was removed with a cloth. Animals with weights less than about 100 g were weighed on Mettler type H6T dig. balance, while larger animals were weighed on a Mettler top-loading precision balance, model P-1200. After completion of a series of weight measurements for an experiment, the shell of each animal was weighed, and the shell weight then subtracted from the total body weight to obtain the wet weight of the soft tissue.

To determine the error due to drying and weighing technique, several animals were kept in 100% sea water and weighed at various time intervals. With large animals (100 g or more) less than 1% variation in body weight was noted over a period of up to 24 hours, but with small animals, water retained on the body surface or in the pallial cavity, led to a variation of $\pm$ 2% in consecutive readings in some of the control experiment measurements. When the animals were placed in 75%, 85% or 115% sea water, variations due to weighing techniques were not significant compared with the change in body weight due to the dilute or concentrated media, but in

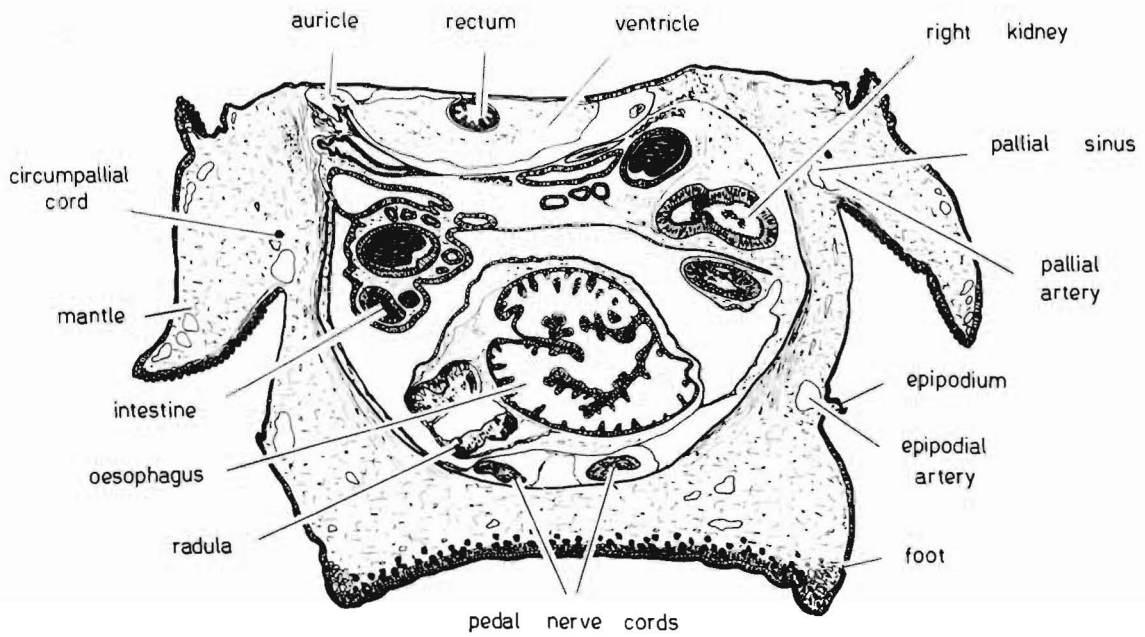

Figure 8: T. S. Scutus showing the position of the epipodial artery from which blood samples were taken. The plane of this section is indicated by line A-B in Figure 1. (The shell has been removed from the dorsal surface.)

experiments where the concentration change in the medium was not so great, errors in weighing are probably the cause of some unevenness in the weight change curves.

2.2.4 Sampling of blood

The epipodial artery (see Fig. 8) was found to be the most convenient vessel from which serial blood samples could be taken. A sample, 0.1-0.2 ml., was collected from this vessel using a disposable hypodermic syringe, fitted with a 25 gauge needle. Determinations of freezing point, and sodium, potassium, calcium and magnesium concentrations of blood samples taken from the epipodial artery were found to be in agreement with analyses made on blood collected from other parts of the body, for example the pedal and cephalic sinuses.

Samples not analysed immediately were stored in small, capped glass tubes at 0-4°C. Under these conditions no significant change in concentrations of cations could be detected after storage for one or two days.

2.2.5 Estimation of osmotic pressure by measurement of the freezing point of the blood

The freezing point depression of a solution is, at fairly low concentrations, directly proportional to the molar concentration of the solution, a one molar solution freezing at -1.86°C. Once the freezing point depression of a solution is known, its osmotic pressure can be estimated by the following equation:

osmotic potential (pressure) $P = 12.06\Delta - 0.02\Delta^2$

where Δ is the freezing point depression of the solution (Harris and Gortner, 1914). However, the absolute

osmotic pressure is temperature dependent.* Results in this work are just expressed as the depression of the freezing point of the sample.

The freezing point (or, more correctly, the melting point) of samples was measured by the microcryoscopic method of Ramsay and Brown (1955). For holding the samples, finely drawn out pyrex tubing was found to be satisfactory; samples were drawn into these tubes immediately before analysis. Identical readings were obtained for samples in pyrex and vitreosol tubing and, as pyrex tubing was much more convenient to draw out, it was used in preference to the vitreosol tubing recommended by Ramsay and Brown. Temperatures were read with a Beckman differential thermometer marked in 0.002°C divisions and calibrated against the melting points of several standard sodium chloride solutions. For six standard samples the experimental error was found to be 0.0003°C.

2.2.6 Estimation of protein

Protein estimations were made using the Kjeldahl method for total nitrogen, and multiplying by the conventional factor of 6.25. Each 1 ml. sample of blood was digested for 30 minutes with 2 ml. of a digestion mixture, which had been prepared by dissolving 1 g of copper sulphate and 1 g of selenium dioxide in 100 ml. of nitrogen-free, concentrated sulphuric acid. After

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* $$p = \frac{g \times (273+t) \times 760 \times 22400}{273\ mv}$$

where v = volume of solvent (ml.)
g = weight of substance dissolved (g)
m = molecular weight
t = temperature (°C)

digestion, 10 ml. of 10N NaOH were added to the mixture, which was then steam-distilled, the steam being allowed to condense into 15 ml. of distilled water, which contained three drops of methyl red indicator (0.03% in 60% propanol) and seven drops of 0.04% aqueous bromocresol green. The solution was titrated with 0.1N HCl, the end-point colour change being from blue to grey to red. Each millilitre of 0.1N HCl, used in the titration was equivalent to 1.4 mg of nitrogen.

Electrophoretic separation of the protein in the blood was carried out in a 2.5-30% polyacrylamide gel. The buffer was a tris-glycine mixture, prepared by mixing 0.5% tris with 0.3% glycine, so that the pH of the solution was 9.1; the voltage gradient was 30 volts per centimetre. After electrophoretic separation some samples were stained with naphthol black to identify the protein bands, and others were subjected to the Mallory haematoxylin staining technique for copper (Lillie, 1965 p. 441) to try to identify the bands which contained copper, which would distinguish haemocyanin from other proteins.

2.2.7 Estimation of cations

All standard solutions were made with AR grade chemicals and dilutions with Elgastat de-ionised water. (Elga specify that the effluent from Elgastat has total dissolved solids not exceeding 2 p.p.m.) Standard stock solutions were kept in polythene bottles. All glassware was thoroughly washed, and then rinsed with de-ionised water before use, except in the analyses of calcium and magnesium by atomic absorption spectrophotometry, when the glassware was soaked in a 1% solution of the disodium salt of ethylene-diamine-tetra-acetic acid (EDTA) before use.

Most of the procedures which have been published for the estimation of cations in blood are for the estimation of cations in the blood of humans or other vertebrates, which has a much higher proportion of protein to cations than does the blood of marine invertebrates such as Scutus. Therefore, the limits set for these methods, due to the presence of protein in the blood, would be expected to be well beyond the conditions in the following analyses of the low-protein blood of Scutus.

Sodium

Determinations were made using an EEL flame photometer. A calibration curve was made using a standard sodium chloride solution with a full-scale reading of 0.5 mM/l. of sodium. Blood and sea water samples were diluted 4000x with de-ionised water. Analyses of 60 artificial sea water samples gave a mean recovery value of 101.5 ± 1.9%, and standards added to blood gave similar recovery values.

Potassium

The EEL flame photometer was also used for potassium determinations. A potassium chloride standard solution was used, full-scale reading 0.25mM, and blood and sea water samples were diluted 200x. The recovery for 60 artificial sea water samples was 99 ± 1.16%

Calcium

In 1960 Willis (1960a) stated, "Although many methods have been described for the determination of calcium in blood serum, they give results which vary over a range of nearly 20 per cent and there is still no agreement as to which method is the most reliable. In general, the values given by the flame photometric methods are 3-4% higher than those obtained from the classical oxalate-

permanganate titration, which in turn are about 10% higher than those given by complexiметric and colorimetric methods...." In recent years methods for calcium analysis using an atomic abosrption spectrophotometer have been developed (Willis, 1960b; David, 1959). Willis reported a recovery of 100% of calcium from blood serum and an error of less than ± 2%. This appeared to be one of the quickest and most accurate methods for the determination of calcium, but at the beginning of this study there was no atomic absorption spectrophotometer available for use. Therefore, in early experiments many methods were investigated, to try to obtain a satisfactory one where reasonably rapid, accurate determinations could be made on small volumes of fluid. These included colorimetric methods, such as that of Spare (1964), in which murexide (ammonium purpurate) dissolved in propylene glycol is the colour reagent. This was found to be a rapid method with high sensitivity, 0.005 ml. of blood or sea water being sufficient for an analysis. However, the presence of copper in the blood was found to interfere with the results. Fruton and Simmonds (1958) stated that, although the manner in which copper was bound to haemocyanin was not clear, it appeared to be less stable than that of iron in the haem proteins. Therefore, it is possible that, under the experimental conditions of Spare's method, the copper is split from the haemocyanin molecule. When calcium was precipitated as oxalate from the blood, the supernatant gave a measurable colour change with murexide, using the method of Spare. Raaflaub (1951) found that copper ions in a concentration as low as 1 p.p.m. led to a measurable colour change with murexide.

Titration with EDTA has been frequently used for estimation of calcium, but with very dilute titrating solutions the end-point is difficult to detect accurately

without the aid of photoelectric apparatus. The use of murexide as an indicator (Schwarzenbach, Biedermann & Bangerter, 1946) was found to be improved by the addition of Naphthol Green B as a screening agent (Knight, 1951). A sharper end-point colour change was obtained with the indicator mixture of calcein and thymolphthalein (Tucker, 1957) as used by Baron and Bell (1957) for the specific titration of serum calcium. However, the end-point was still slightly drawn out, possibly because of the presence of phosphate (Schwarzenbach, 1957) and, also, the EDTA-complexing reaction is rather slow (Lewis and Melnick, 1960). The method of Baron and Bell was used for estimations of calcium in preliminary experiments, but it was not used in obtaining any of the results which are quoted in this work.

Results which are given in this section were obtained either by the flame photometric method of Fawcett and Wynn (1961), or with an atomic absorption spectrophotometer.

(a) The flame photometric method of Fawcett and Wynn

In the flame photometer, emission readings of calcium are affected by other substances, particularly sodium and phosphate, present in the blood. Sodium enhances, while phosphate depresses, the emission, the inhibitory action of phosphate being prevented in part by the presence of protein (Chen and Toribara, 1953). Many methods for overcoming interference during calcium determinations in blood with a flame photometer have been published. Most of these require the separation of calcium from the blood (e.g. precipitation as oxalate), but with samples of 0.1 ml., or less, this separation is impractical and creates large errors.

In the 'Calcium Release Method' of Fawcett and Wynn,

samples are diluted with a solution containing 10 mM $MgSO_4$ and 2 mM NaCl. These added ions act as radiation buffers and prevent the interference of the sodium and the phosphate in the sample. A standard stock solution of 75 mM NaCl, 5 mM KCl and 5 mM $CaCl_2$ was diluted 100x with the 10 mM $MgSO_4$ - 2 mM NaCl diluting solution to make up a working standard. Blood samples were diluted 200x with the diluting solution. Readings were made on an EEL flame photometer with a calcium interference filter, and the zero setting was made with the diluting solution.

(b) The atomic absorption spectrophotometric method

When it became available, a Techtron AA4 atomic absorption spectrophotometer was used for calcium analyses. To overcome any protein or phosphate interference, samples were diluted 200x with a 1% solution of EDTA (see Willis, 1960b for discussion). With the addition of EDTA, 100% recovery of calcium is achieved in this method.

Settings of the AA4:

High tension - 400V.
Slit width - 100 μ
Flame - nitrous oxide / acetylene
Air pressure - 15 lbs / $in.^2$
Lamp current - 15 mA.
Wavelength - 4227 Å

Readings were recorded on a chart recorder, and a standard calibration curve was made using dilution of a standard solution containing 450 mM NaCl, 10 mM KCl, 10mM $CaCl_2$ and 50 mM $MgCl_2$ per litre.

Results of analyses of 50 samples by methods (a) and (b) for calcium determination were found to agree closely.

Magnesium

Many procedures have been published for the determination of magnesium in blood. In a comparative study on the performance of several methods (two ammonium phosphate precipitation methods, a Titan Yellow colorimetric method, and EDTA titration) Butler, Forbes, Munroe and Russel (1964) found that they obtained most concordant and relatively precise results with one ammonium phosphate and the EDTA method. However, as with calcium determinations, the small sample size here made precipitation methods unsatisfactory, and with very dilute (0.001 M) EDTA solutions the end-point colour change in the EDTA method is not very sharp. Even so, an EDTA method, with Eriochrome Black T as an indicator (Flaschka, 1959), was very useful in doing isolated checks on magnesium concentrations, particularly on large sea water samples.

Until the atomic absorption spectrophotometer was ready for use, magnesium concentrations in series of samples were determined using Spare's (1962) modification of the colorimetric Titan Yellow (=Clayton or Thiazole Yellow) method, with polyvinyl alcohol as a stabilising agent and colour intensifier. Optical densities were read at 540mμ on a 'Spectronic 20' spectrophotometer.

A method using the atomic absorption spectrophotometer was found to be the most satisfactory method for magnesium determinations. It was rapid, accurate and gave 100% recovery on samples which had been diluted 1000x. (The atomic absorption spectrophotometer is sensitive to magnesium at levels of about 0.5 p.p.m.) As with calcium estimations made using the AA4, dilutions of samples for magnesium analysis were made with

a 1% EDTA solution, to overcome protein and phosphate interference (Willis, 1960c). A set of replicate samples gave a variation of less than ± 1%. The same standard stock solution containing sodium, potassium, calcium and magnesium chlorides, which had been used as a calcium standard, was also used in the magnesium method to obtain a calibration curve. Settings of the AA4 were the same as for the calcium determinations, except that the wavelength setting for magnesium measurements was 2852Å.

Although magnesium measurements made on sea water samples by the Titan Yellow method and the AAS method were found to be in agreement within the range of experimental error, the AAS method gave consistently higher values for magnesium content of the blood than were obtained using the Titan Yellow method. It was assumed that the protein content of the blood was the cause of the lower magnesium values in the colorimetric method, even though the protein content of the blood was less than one per cent. Magnesium determinations by both the Titan Yellow and the AAS methods were made on a series of 50 blood samples taken from animals in different dilutions of sea water. Analyses of these results showed that the values for magnesium concentrations obtained by the Titan Yellow method could be multiplied by a constant factor to correct them, within the range of experimental error, to those obtained by the AAS method. All of the determinations of magnesium by the Titan Yellow method were corrected by this factor.

2.2.8 Estimation of anions

Chloride ions are easily the most abundant anions present in sea water and in the blood of marine invertebrates, usually accounting for about 95% of the inorganic ions present. (Many artificial physiological

media are made solely from chlorides.) It is to be expected that, if cation concentrations and the total osmotic concentration of the blood of an animal change, the chloride concentration will change in a similar fashion. The chloride concentration of the blood of a marine invertebrate is usually in equilibrium with that in the surrounding sea water (see, e.g., Robertson, 1964).

It was thought that it would be of interest to determine the concentration of sulphate in the blood of Scutus, and compare it with the concentration of sulphate in the sea water, because the concentration of sulphate in the body fluids of marine invertebrates has often been shown to differ from the concentration in the surrounding sea water.

As has been already mentioned, phosphate is known to cause interference in the analysis of calcium by flame photometric methods, and analyses of blood phosphate were made in the first instance to see if the concentration was great enough to be likely to interfere with the measurement of calcium by a flame photometric method. Another reason for interest in the phosphate level of the blood was that experiments planned for a later part of this study involved preparation of artificial physiological solutions with ionic concentration similar to the blood. Saline solutions sometimes include phosphate as a buffer, and it would therefore be advantageous to know the actual concentration of phosphate in the body fluid.

Sulphate

The most satisfactory method for the estimation of sulphate was found to be one based on the volumetric benzidine method of Power and Wakefield (1938). Using this method, the sample (0.1 ml.) was deproteinized with trichloroacetic acid and the sulphate was precipitated

with 5 ml. of a 1% solution of benzidine in 95% ethyl alcohol. After the precipitate had been dissolved in hot water, sulphate was estimated by titration from a 1 ml. burette with 0.005N NaOH solution, using aqueous phenol red as indicator. This method gave a mean recovery of 100.7 ± 2.3% of known additions of sulphate to a sample. The iodate method of Webb (1939) and the colorimetric diazotized benzidine method of Milton and Waters (1949), modified after Cuthbertson and Tompsett (1931), gave similar levels of sulphate in blood and sea water, but the experimental error was found to be greater with these methods.

Phosphate

The method used for phosphate determinations was that of Horwitt (1952). Protein was removed by precipitation with trichloroacetic acid, ammonium molybdate solution was added to the filtrate and the phosphomolybdate then reduced with a very weak solution of stannous chloride in hydrochloric acid; the absorbance of the resulting solution was read in a 'Spectronic 20' spectrophotometer at 660 mμ, which was the maximum transmittance of the recommended filter for the blue solution. Standard solutions were prepared from potassium dihydrogen phosphate.

2.3 RESULTS

All experiments in sections 2.3.2, 2.3.3 and 2.3.4 were carried out at room temperature. The temperature of media in the aquaria during these experiments was found to range from 17-19°C. Results from a few experiments conducted in a controlled temperature room at 7°C are given in section 2.3.5.

Standard deviation ranges have not been included in

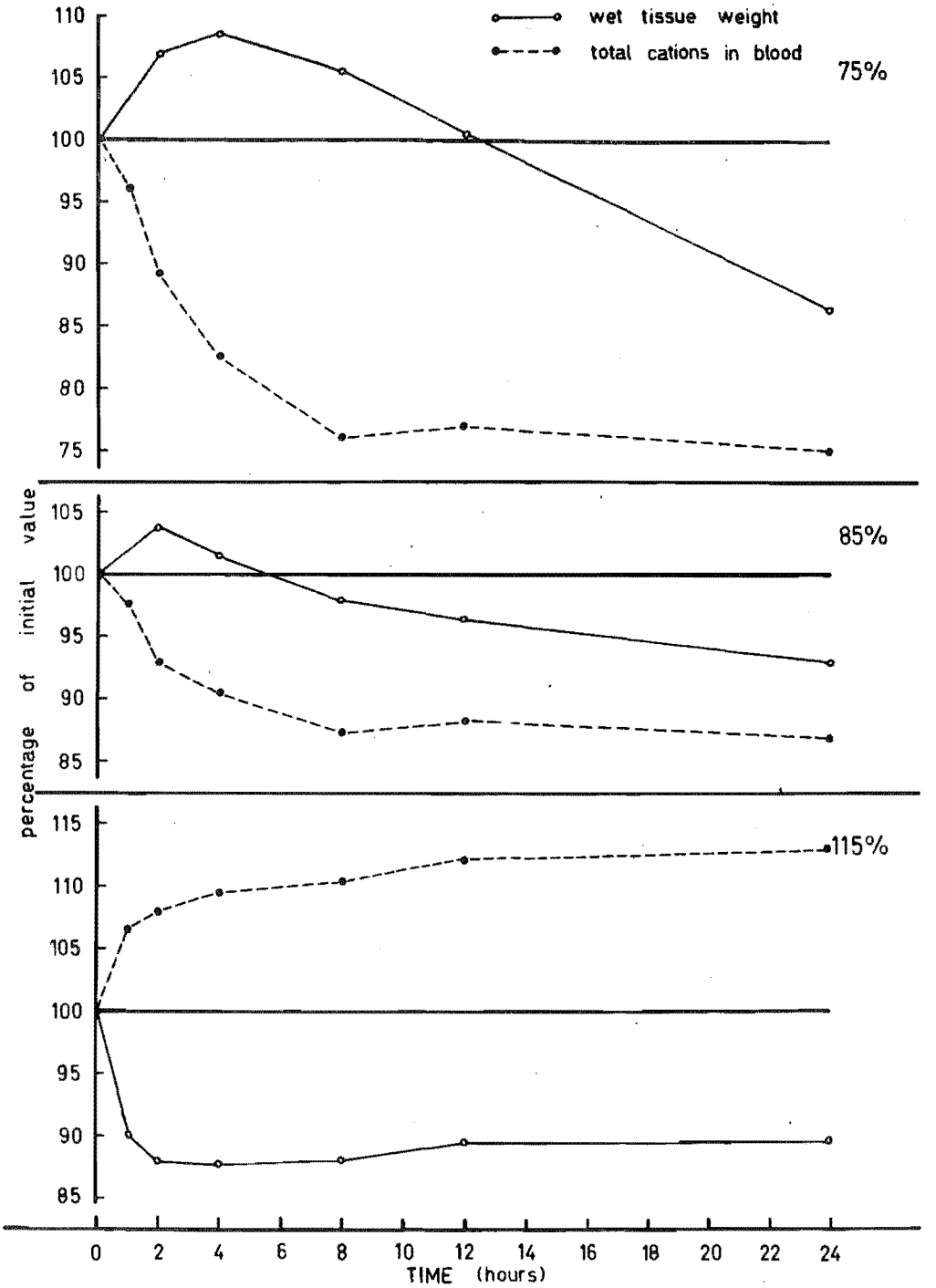

Figure 9: Mean values of wet tissue weight and total cations (sodium + potassium + calcium + magnesium) in the blood of animals placed in

(a) 75% sea water (4 animals)
(b) 85% " " (6 ")
(c) 115% " " (3 ")

figures showing curves of the mean body weight and mean blood concentration changes of animals placed in experimental media. The rates at which these changes occurred varied considerably depending on the sizes of the animals. For measurements made during the first few hours after animals had been placed in the experimental media, the size of the standard deviation of the mean was directly related to the range of sizes of the animals used. Therefore, rather than try to represent this variability by including standard deviation ranges, in addition to figures showing the curves of mean values, figures for curves for all animals, showing individual body weights, have been included.

2.3.1 Weight changes of animals in media of different concentrations

(a) Hypotonic media

75% sea water Animals placed in 75% sea water showed a rapid increase in body weight accompanied by a noticeable loss in muscle tone. Immediately after transfer to the diluted medium animals usually showed a short period of increased activity but, as the animals became more obviously oedematous and the muscle tone decreased, movement almost stopped. In some cases animals were unable to maintain their grip on the walls of the aquarium and turned upside-down. As the condition of the animals appeared to deteriorate, large quantities of mucus were often secreted and animals showed a tendency to lose their black pigmentation. With one small animal (35 g) this loss of pigmentation was observed after it had been four hours in the diluted medium, but with other large animals pigment loss was not noticeable until after about twelve hours.

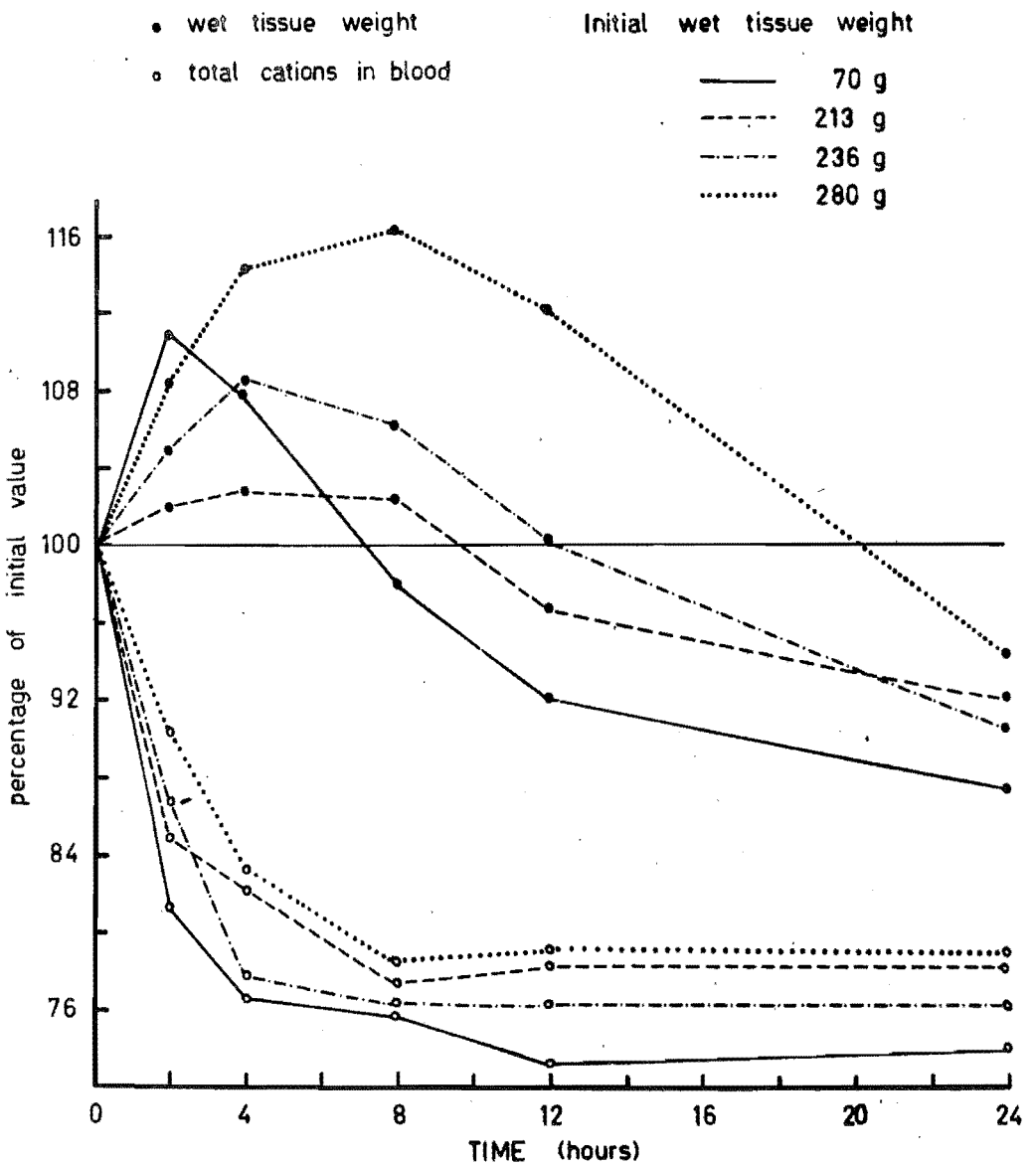

Figure 10: Wet tissue weights and total cation concentrations in the blood, of four animals placed in 75% sea water.

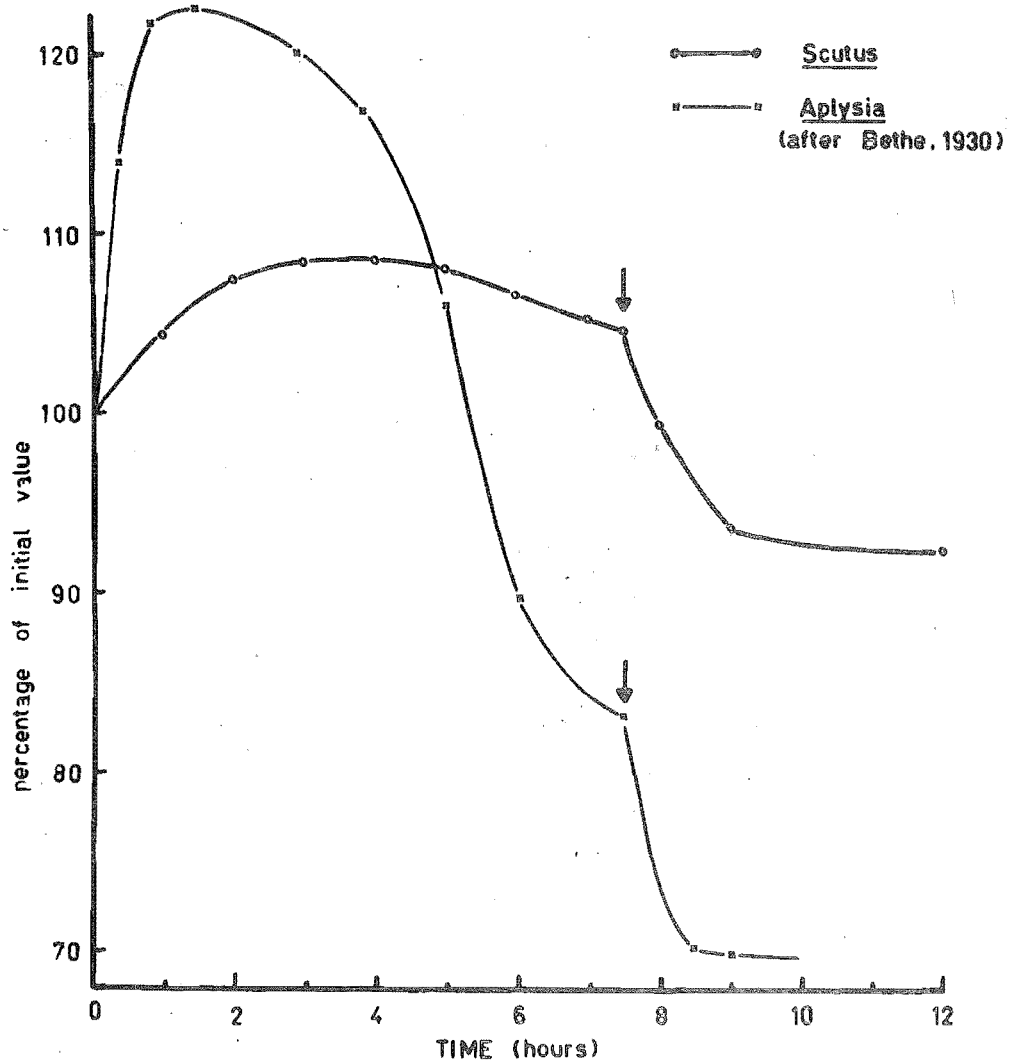

Figure 11: Weight change of animal left in 75% sea water for seven and a half hours and then returned to normal sea water (at arrow). The weight change curve for Aplysia when subjected to similar treatment (Bethe, 1930) is also shown in the figure.

A curve showing the mean wet tissue weight change of four animals over a period of 24 hours in 75% sea water is shown in Figure 9; the weight change curves of the individual animals are shown in Figure 10. This latter figure illustrates that both the degree of increase in weight and the time taken for the animals to reach their maximum weights varied between animals. After a variable period (sometimes after as little as two hours or, with other specimens, not until they had been in the dilute medium for eight hours or more), a decrease in body weight was shown. This decrease continued fairly rapidly in 75% sea water and eventually resulted in the death of the animal.

If animals were replaced in 100% sea water, after being in 75% for a few hours, they showed a rapid decrease in weight and increase in muscle tone. Figure 11 shows the weight change curve of one animal left in 75% sea water for seven and a half hours and then returned to normal sea water. After the initial rapid weight decrease on return to the normal medium, the curve showing the body weight began to level out at a little less than the initial value in 100% sea water, indicating that some salts had been lost during the time in which the animal was in the dilute medium. After a period of four hours in 75% sea water, some animals did not recover when returned to normal sea water, but continued to lose weight and eventually died. Other animals were able to withstand a longer period in the dilute medium. The critical period appeared to be related to the size of the animal, the larger the animal the longer it could tolerate subjection to the dilute medium.

85% sea water Changes similar to those which occurred when animals were placed in 75% sea water were shown by animals in 85% sea water, but the increase in body

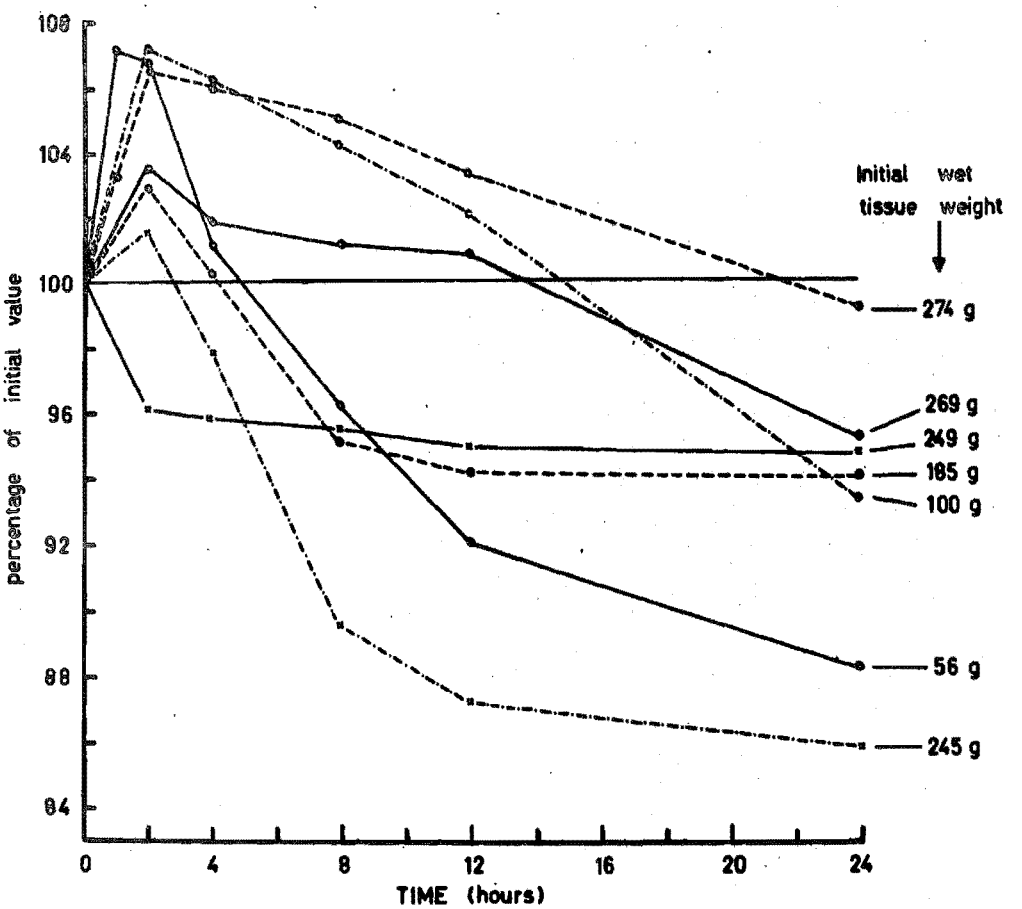

Figure 12: Wet tissue weight changes of eight animals placed in 85% sea water.

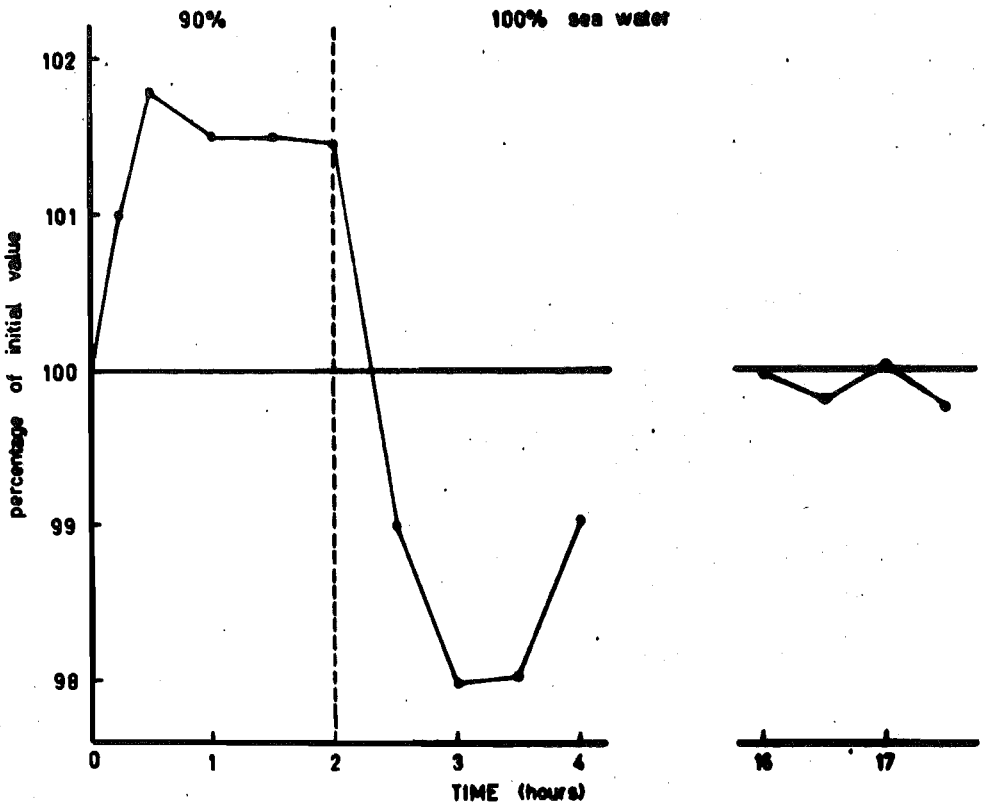

Figure 13: Wet tissue weight changes of animal placed in 90% sea water for two hours and then returned to normal sea water.

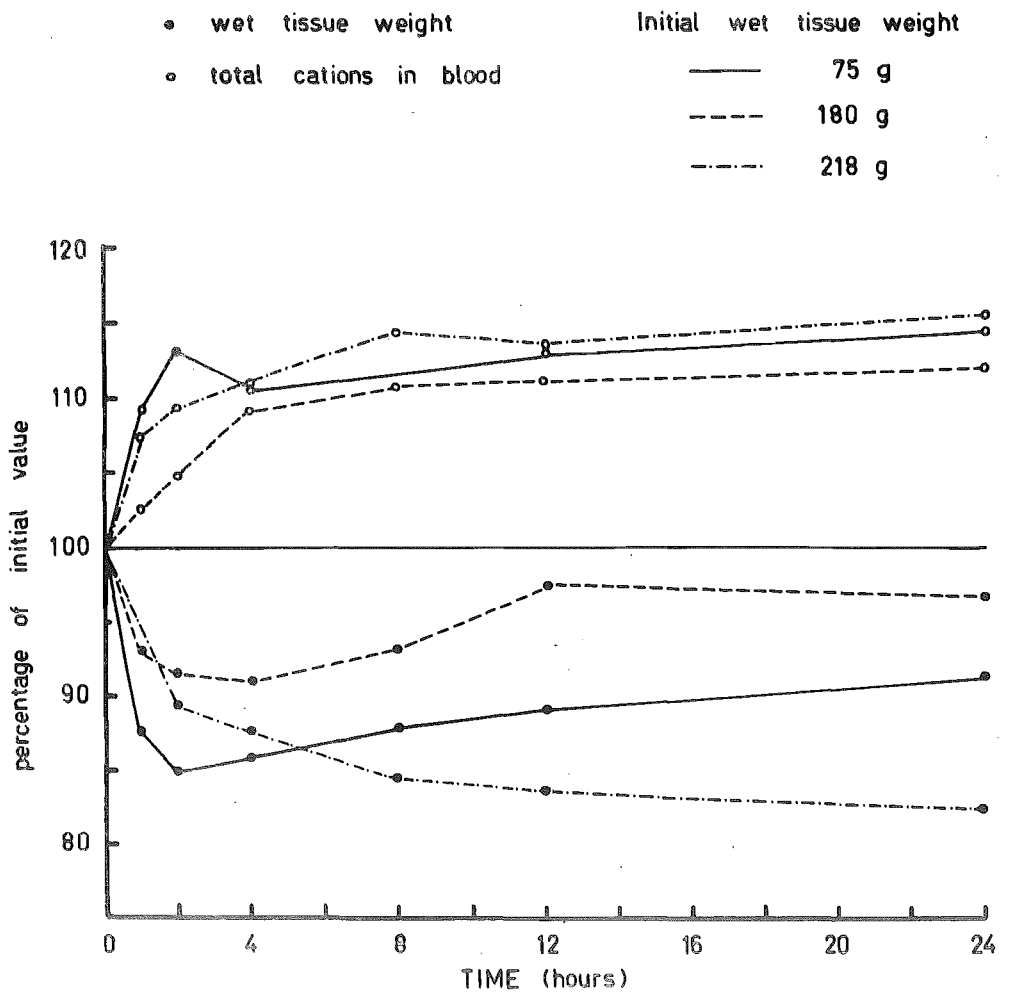

Figure 14: Wet tissue weights and total cation concentrations in the blood, of three animals placed in 115% sea water.

weight was not so large, the movement of the animals was greater, and they could survive for a longer period in this medium. Figure 9 shows the mean wet tissue weight changes for six animals placed in 85% sea water. Figure 12 illustrates that the degree of change of body weight was very variable and that the size of the specimen had an effect on the rate of change of body weight. With the exception of the animal with initial weight 245 g in normal sea water, the degree of decrease in body weight after 24 hours in the dilute medium was inversely proportional to the initial body weight. As with the animals placed in 75% sea water, the length of time that the animal could survive in the dilute medium was correlated with the body size.

90% sea water When animals were placed in 90% sea water for two hours they showed an increase in weight; when returned to normal sea water they showed a decrease below the initial weight and, after about an hour or more in normal sea water, an increase in wet tissue weight, eventually returning to approximately the original body weight (Fig. 13).

(b) Hypertonic medium (115% sea water.)

Figure 9 shows the mean values of weight changes, and Fig. 14 individual weight changes, for three specimens when placed in 115% sea water for a period of 24 hours. In this medium the animals showed normal movement and maintained good muscle tone throughout the period of experiments, which extended up to five days. They showed an initial weight decrease and then, after about eight hours, they usually slightly increased in weight and then maintained a fairly constant weight, at about 90-95% of the initial value in normal sea water.

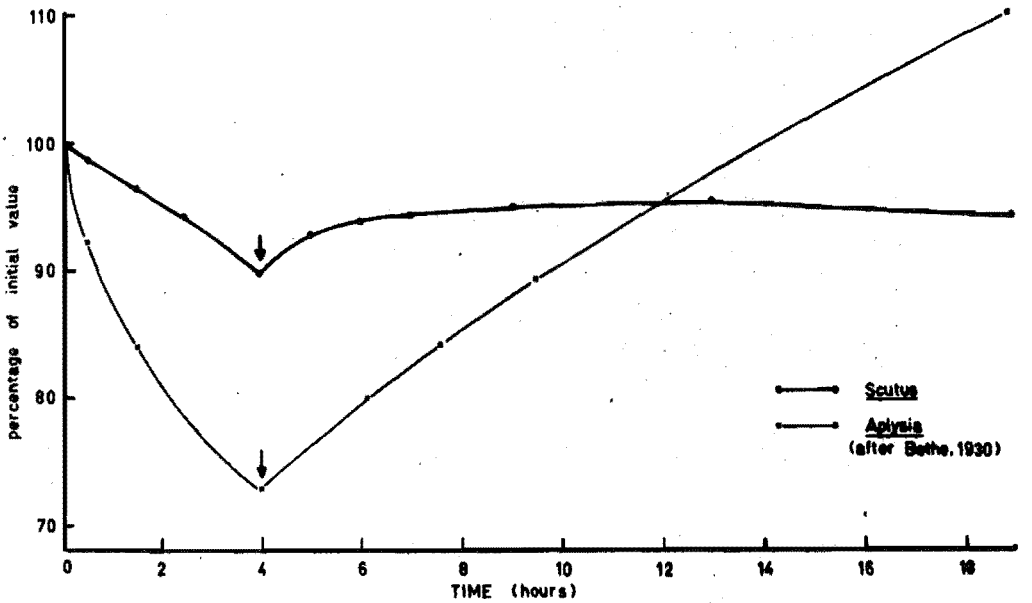

Figure 15: Weight change of one Scutus specimen placed in a medium of three parts normal sea water and one part isotonic sucrose solution for four hours, and then returned to normal sea water (at arrow). Also shown in the figure is the weight change curve for Aplysia under similar conditions (after Bethe, 1930).

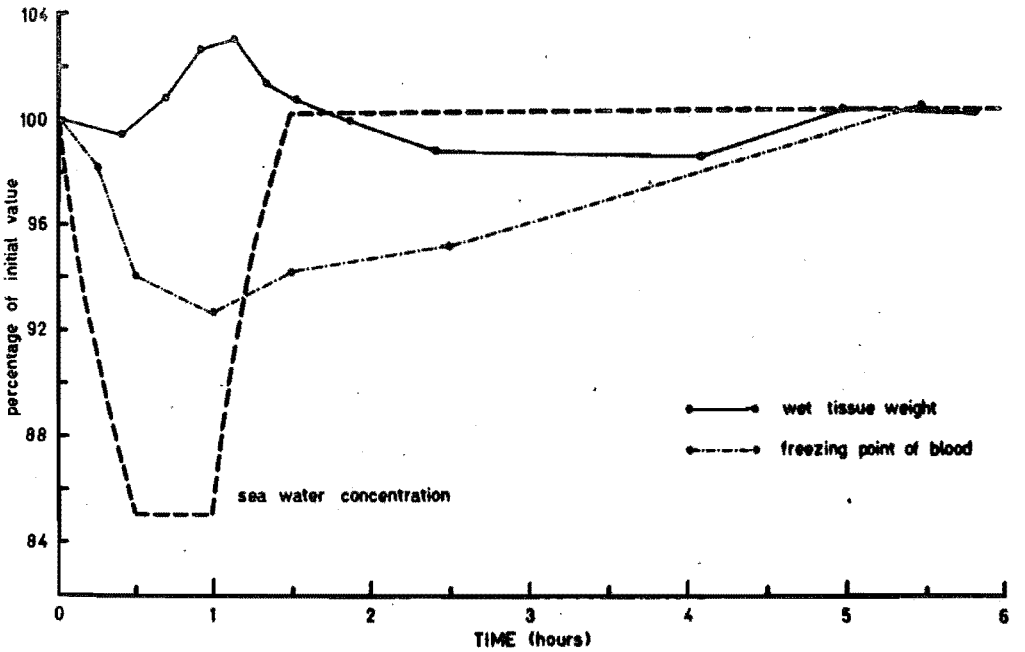

Figure 16: Change in wet tissue weight (one animal) and freezing point of blood (mean 16) when animals subjected to changing sea water concentration simulating estuarine environmental conditions.

(c) Three parts sea water / one part isotonic sucrose solution

As had been shown for Aplysia by Bethe (1930), animals placed in a medium of three parts normal sea water and one part isotonic sucrose solution showed a decrease in body weight. Figure 15 shows the weight change curve for one specimen of Scutus compared with that given by Bethe for Aplysia. Upon return to 100% sea water the weight increased; Scutus did not regain its initial weight in normal sea water, while Aplysia showed an increase to 110% of the initial body weight.

(d) Simulation of environmental conditions at the Heathcote-Avon Estuary

When sea water was diluted to 85% concentration over a period of 30 minutes, kept at this concentration for half an hour and then returned to its original concentration over a further 30 minute period, animals showed a slight, but definite change in body weight. Figure 16 shows the weight change curve for one animal. Several animals were used and the changes in all were essentially the same, i.e. all animals showed a slight increase in weight when the water was diluted, followed by a weight decrease when the concentration of the sea water returned to its normal value. The time at which the maximum body weight was reached lagged a little behind that at which the water was at its minimum concentration. There was a tendency for the weight to drop a little below the initial value upon the return of the sea water to its 100% concentration, but within about three hours the animal had regained approximately its original weight. The weight change of one animal only is shown, because the time taken for animals to reach maximum and minimum values varied slightly from animal to animal; therefore, plotting the mean values of weight changes of several animals

would mask the characteristic individual curve with its slight, but definite, maximum and minimum.

2.3.2 Composition of sea water

Mean values obtained for the concentrations of sodium, potassium, calcium, magnesium and sulphate ions in several samples of 100% sea water (i.e. sea water collected from Lyttelton), and the mean freezing point value, are shown in Table 3.

TABLE 3

ion	mean value mM / l.	standard deviation	N
Sodium	487	33.14	14
Potassium	10.87	0.67	14
Calcium	11.56	1.09	14
Magnesium	47.99	4.07	14
Sulphate	17.80	1.05	5
Freezing Point	-1.87°C	0.107	14

It can be calculated from Barnes' (1954) sea water tables that the sea water at Lyttelton has a chlorinity close to 19.5°/oo, and salinity close to 35°/oo. The calcium concentration shows relatively more variation than the other cations, and the proportion of calcium in the Lyttelton sea water is slightly greater than that given by Barnes for open sea water, but this is to be expected for water that has been collected inshore.

TABLE 4

Concentrations of ions in sea water collected from Lyttelton and ratios of constituents in the blood to the external medium.

ion	mean value for sea water mM/l.	mean value for blood mM/l.	Ratio of blood constituents to sea water		N.
			mean	S.D.	
sodium	487	490.9	1.008	0.043	20
potassium	10.87	11.26	1.036	0.040	34
calcium	11.56	11.94	1.033	0.044	31
magnesium	47.99	49.19	1.025	0.098	24
sulphate	17.80	17.57	0.987	0.028	7
phosphate	*	0.258±.001			8
protein	*	0.985±.28			6
freezing point	-1.87°C	-1.89°C			

* No analyses made

Magnesium and sulphate concentrations were found to be slightly lower than values given by Barnes for the open sea.

2.3.3 Ratios of blood constituents to the medium in normal (100%) sea water

The results shown in Table 4 were obtained by analysing the blood of animals which had been kept for at least 48 hours in sea water collected from Lyttelton. In some preliminary experiments a few animals were kept in water collected either from the estuary, at a time when it was not appreciably diluted, or from Scarborough. Ratios of concentrations of constituents in the blood of these animals, compared with those in the external medium (sea water from the estuary or Scarborough), were found to fall within the same range as those of animals kept in sea water collected from Lyttelton. The concentrations of sodium, potassium, calcium, magnesium and sulphate ions in the blood were all very close to those of the sea water in which the animals had been equilibrated. Phosphate and protein were both present in the blood in only low concentrations.

Electrophoretic separation of the blood protein gave one very dense band (stained with naphthol black), and several faint bands, all in the globulin region. The main protein band gave a positive reaction to the Mallory staining technique for copper, indicating that it was haemocyanin. The faint bands could perhaps be dissociation products of haemocyanin for, on either side of the isoelectric point, it easily dissociates into particles (Brosteaux, 1937; Eriksson-Quensel & Svedberg, 1936; Boeri, 1963); the buffer used during the electrophoretic separation had a pH considerably higher than the isoelectric point of haemocyanin.

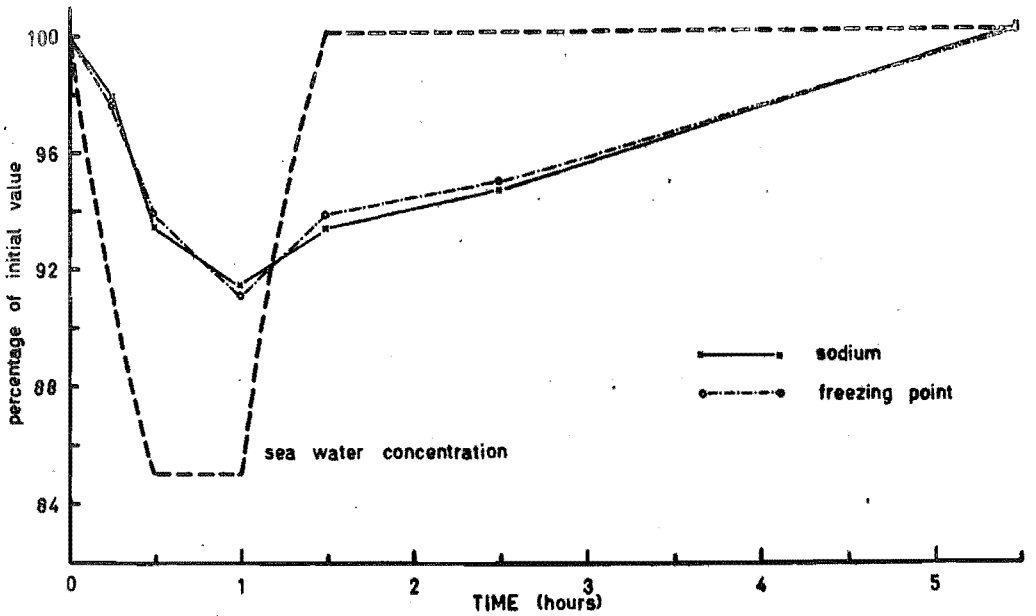

Figure 17: Change in sodium concentration and freezing point of the blood of animals subjected to changing sea water concentration simulating environmental conditions. (Each curve mean of values for 12 animals).

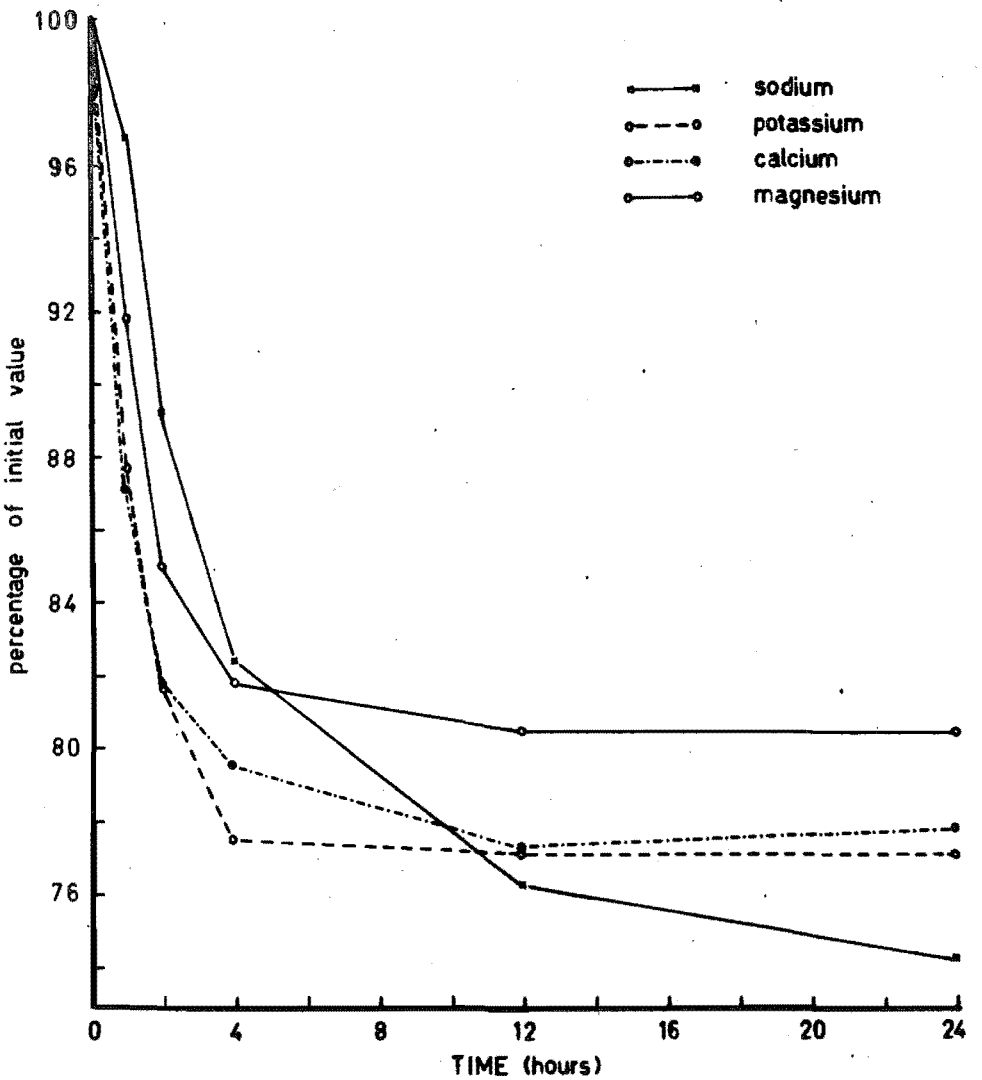

Figure 18: Changes in concentrations of cations in the blood of animals placed in 75% sea water. (Each curve mean of values for four animals).

2.3.4 Freezing point depression, and cation concentration changes of the blood

The percentage changes of the freezing point of the blood were found to parallel those of the sodium concentration so closely (see Fig. 17) that, in later experiments, only occasional freezing point determinations were made.

(a) Hypotonic media

75% sea water Changes in the total cation concentration (sodium + potassium + calcium + magnesium concentrations) of the blood of four animals placed directly into 75% sea water are shown in Figure 10. Mean values for these four animals are shown in Figure 9. There was an immediate and sharp decrease in the cation concentration and, after a period, which might be as short as four hours for small specimens, the cation concentration of the blood was almost the same as that of the surrounding medium. This concentration then remained fairly constant.

The initial rates of change of concentrations of each of the four cations were similar, but after about four hours the decrease in magnesium was less marked, and that of sodium more rapid, than those of the other ions (Fig. 18). However, the differences are not thought to be of physiological significance since, by this stage, the animals appeared to be in very poor physical condition.

It was noted that a lessening in the rate of decrease of the cation concentrations occurred at approximately the same time (after about four hours in the diluted medium) as the body weight began to decrease after its maximum value (Fig. 9).

Samples taken 24 hours after an animal had been

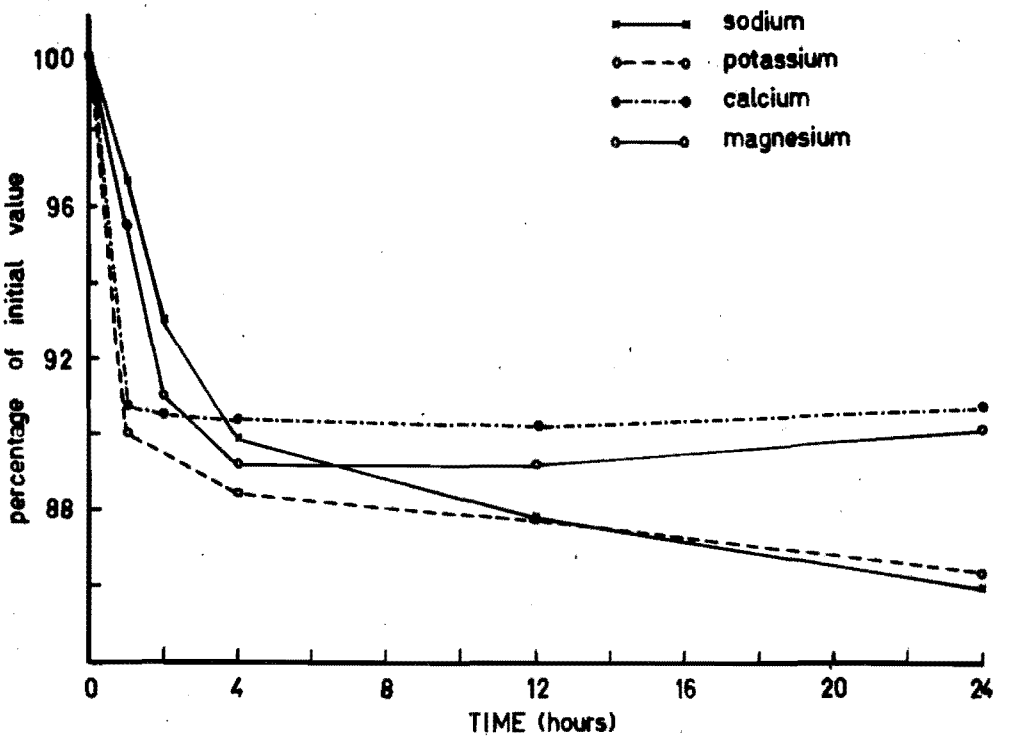

Figure 19: Changes in concentrations of cations in the blood of animals placed in 85% sea water. (Each curve mean of values for six animals).

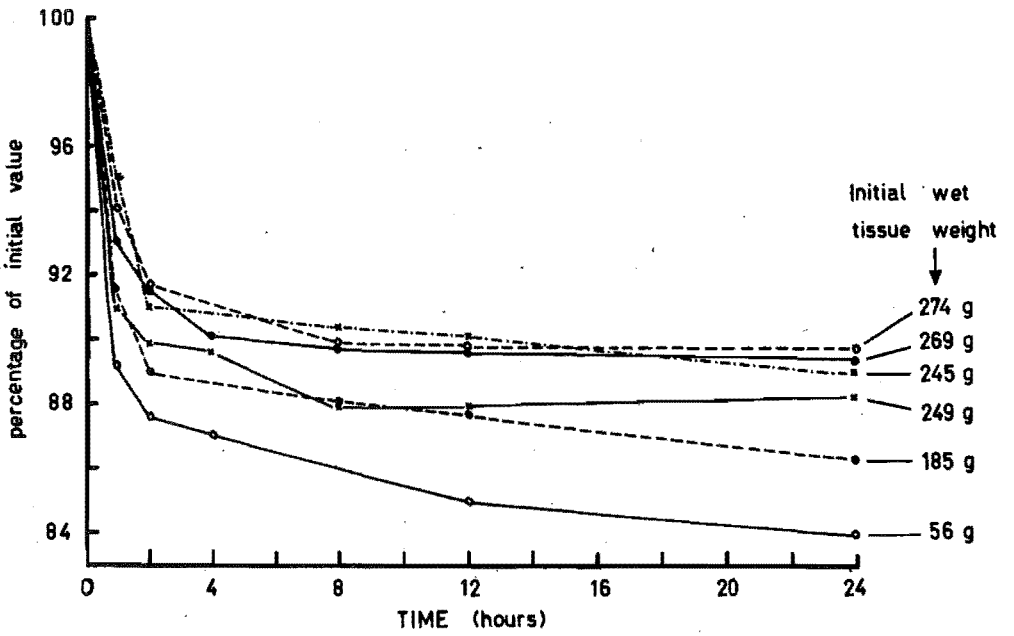

Figure 20: Changes in the concentration of total cations (sodium + potassium + calcium + magnesium) in the blood of animals of different size when placed in 85% sea water.

returned to normal sea water, after having been seven and a half hours in 75% sea water, showed that the cation concentrations had returned to approximately their initial values - for two animals the mean total cation concentration was 100.4% of the initial value in normal sea water.

85% sea water Total cation concentrations changes of the blood of animals in 85% sea water showed the same trends as those of animals kept in 75% sea water, but the dilution of the blood was not so rapid. However, eventually total cation concentrations in the blood were very close to those of the external medium. Fig. 9 shows the curve for the mean total cation concentration change for six animals, and Fig. 19 the changes in the individual cations. At the 5% probability level, the decrease in the calcium ion concentration after 24 hours in the dilute medium was significantly less than that of sodium, but the differences between the other ions were not significant.

The size of the animal had a marked effect on the rate of dilution of the blood and this is illustrated in Figure 20, which shows the changes in total blood cation concentration for six specimens with weights ranging from 56 g to 274 g, after the animals had been placed in 85% sea water.

(b) Hypertonic medium (115% sea water)

The cation concentration changes in the blood of animals kept in 115% sea water were determined for only three animals, but in each case a similar increase in cation concentration was shown. After about twelve hours in the concentrated medium the total cation concentration remained at a fairly constant level, a little below that of the external medium (Fig. 9). This level was maintained for four days, when the experiment was terminated.

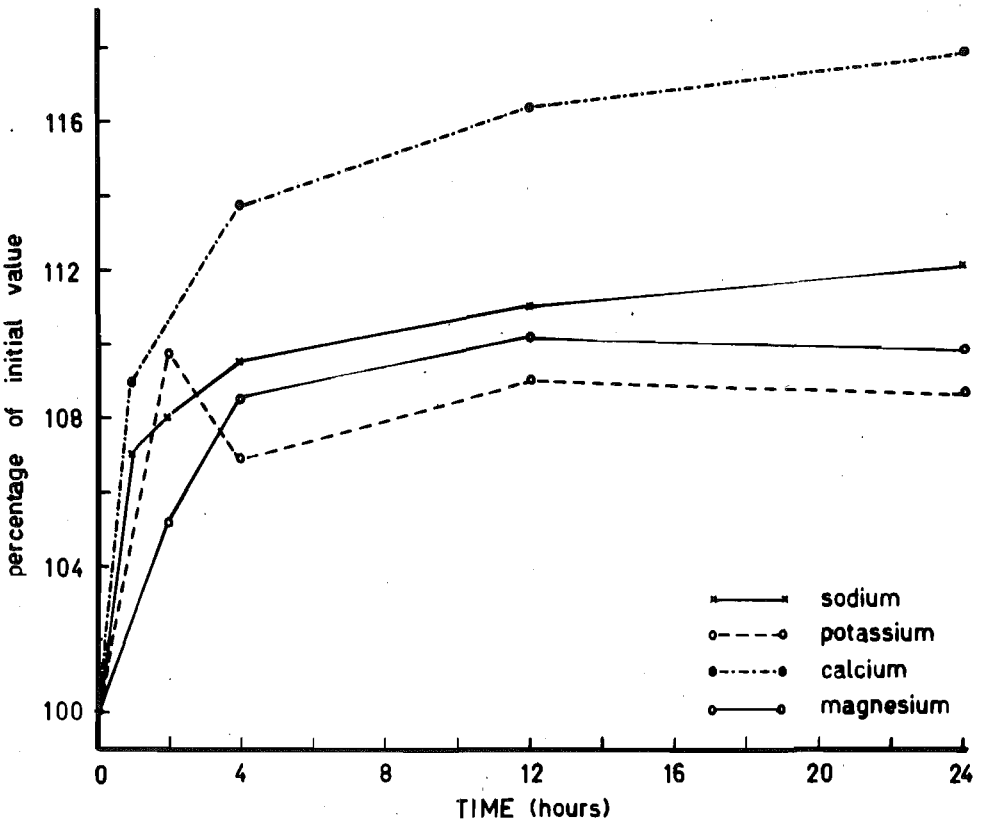

Figure 21: Changes in the concentrations of cations in the blood of animals placed in 115% sea water. (Each curve mean of values for three animals).

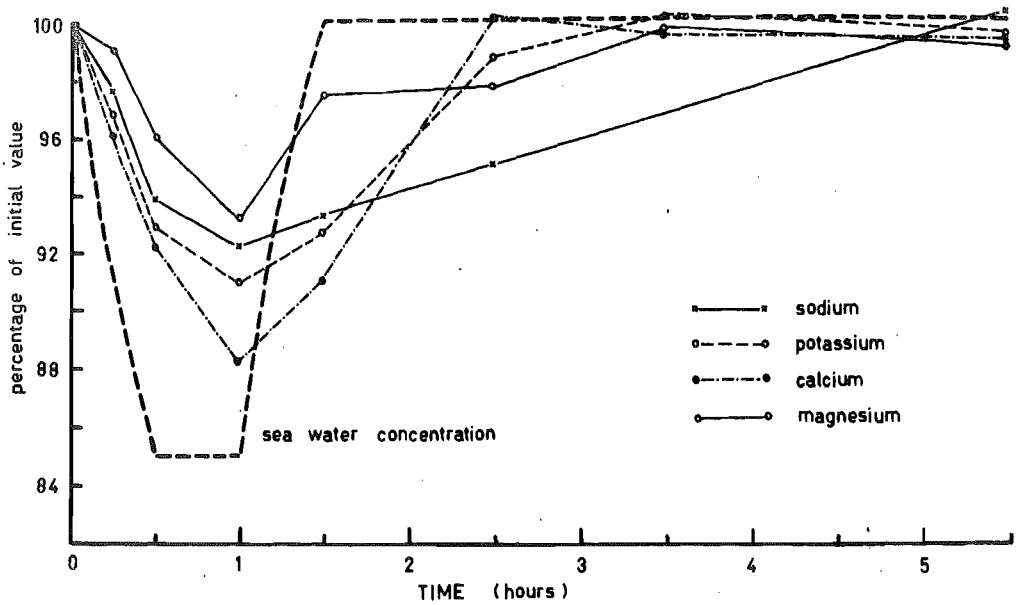

Figure 22: Changes in the concentrations of cations in the blood of animals subjected to changing sea water concentration simulating environmental conditions. (Each curve mean of values for 22 animals).

Analysis of results obtained from these three specimens showed that calcium increased to a significantly greater level than the other cations (using calcium and potassium values $P < 0.025$) (Fig. 21).

(c) Three parts sea water / one part isotonic sucrose solution

After four hours in this medium there was a decrease in the total cation concentration of the blood to 90% of the value in 100% sea water. This was not as great a decrease in concentration as occurred within the same time in the blood of animals kept in 75% sea water.

(d) Simulation of environmental conditions at the Heathcote-Avon Estuary

As was expected from initial weight change experiments (Fig. 16), animals subjected to gradual dilution of the sea water to 85% for a short period showed a slight decrease in the concentrations of various cations (Fig. 22) and then these returned to close to their initial values in normal sea water when the medium was restored to its original concentration. The minimum concentration measured for each cation was reached one hour after the dilution was commenced, just before the medium was concentrated again. At this time the calcium concentration was significantly less than the magnesium and sodium concentrations ($P < 0.05$), but it was not significantly less than the potassium concentration. The concentrations of ions other than calcium were not significantly different from one another at the 5% probability level.

The variation in the osmotic pressure of the blood, as measured by freezing point depression, closely followed this pattern also, the curve for the freezing point change being almost identical to that of the sodium ion change (compare Figures 16 & 22).

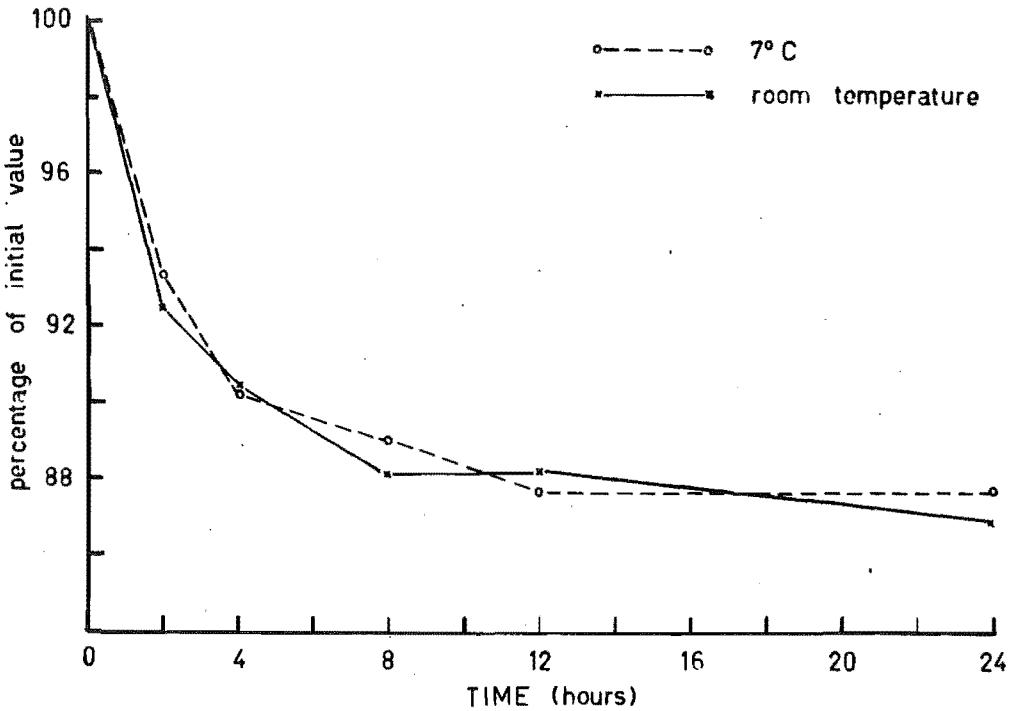

Figure 23: Changes in the total concentration of cations (sodium + potassium + calcium + magnesium) in the blood of animals placed in 85% sea water at 7°C and at room temperature (17-19°C).

2.3.5 Effect of temperature of medium

(a) Animals in 85% sea water

When two animals were kept in 85% sea water at 7°C, the muscle tone appeared to remain firmer than those kept at room temperature (17-19°C). However, a similar initial weight increase, and subsequent decrease, was found. The cation changes in the blood of the two animals in the dilute medium at 7°C were found to be not significantly different ($P > 0.5$) from those at room temperature. Mean total cation concentration changes for two animals at 7°C and four animals at room temperature (17-19°C) are shown in Fig. 23.

(b) Animals in sea water of varying concentration as found at the Heathcote-Avon Estuary

As in experiments on animals put directly into 85% sea water, some experiments on animals in a medium of variable salinity, imitating salinity changes at the estuary, were conducted in a temperature-controlled room at 7°C. Again, although the animals appeared to have better muscle tone than those kept at room temperature, there was no significant difference in the rate of change of the cation concentration of the blood. The mean of the total cation concentration did not decrease below 90% of the initial value.

2.4 DISCUSSION

2.4.1 Composition of the blood of Scutus in normal sea water

As the characteristic state of a living organism is that of an open system (von Bertalanffy, 1950), there must be a continuous exchange of materials between the organism and the environment. Therefore, from a physical point of view, even after an animal has been living for an extended period in normal sea water, it cannot be said to

be in true equilibrium with the medium, but in a steady state. Individual ionic concentration differences between the blood of marine molluscs and the surrounding medium are widespread, even though the blood plasma is in osmotic equilibrium with sea water (at least within 1-2%) across the permeable membranes of the animal. The slight differences between concentrations of the ions in the blood and those in the sea water could be brought about by one or more of the following:

1) Donnan effects, due to the presence of protein in the blood. However, Prosser (in Prosser and Brown, 1961) states that protein-binding is insignificant in marine animals; for marine molluscs this statement is substantiated by the fact that most of the protein in the blood of molluscs is in the form of haemocyanin (Ghiretti, 1966) and this has a very low binding capacity for cations (Webb, 1940). The total protein concentration in the blood of Scutus is low and, from electrophoretic studies, appears to be mostly in the form of haemocyanin; therefore, protein binding may be expected to be slight in Scutus.
2) differential degrees of permeability of the body wall of the animal to various ions,
3) active excretion of salts and water, or
4) active uptake of salts and possibly water.

In this study no analyses were made of cation concentrations in blood dialysed against sea water, so it is impossible to come to firm conclusions about ionic regulation in Scutus. However, mean values of concentrations of cations in the blood of Scutus do show the typical molluscan trend, in that sodium concentrations in the blood and sea water are almost exactly the same, while calcium, potassium and magnesium are a little

more concentrated, and sulphate a little less concentrated in the blood than in the surrounding sea water.

2.4.2 Effects of variation in the concentration of the external medium

Changes in concentration of the blood were followed by taking serial samples from animals. Very little work has been done on the blood volumes of marine prosobranch Gastropoda, but Brown (1964) made an extensive study on the blood volumes of three species of Bullia and obtained values that ranged from 49.3 to 75.3 ml. blood / 100 g wet tissue weight. Pilson (1965) calculated the blood volume of Haliotis fulgens to be about 41%. Martin, Harrison, Huston and Stewart (1958) gave blood volume figures for the opisthobranchs Aplysia and Archidoris of 79.3% and 65.4% respectively. It would seem likely that Scutus would have a blood volume of at least 40%. Therefore, taking 0.1-0.2 ml. samples from animals with blood volumes probably never less than 20 ml., and more likely to be nearer 60-80 ml., would not be expected to cause much stress to the animal, as far as the effect of sampling on the blood volume is concerned.

It has been noted many times (e.g. see Lafon, 1953) that most marine invertebrates are poikilosmotic, the concentrations of the body fluids changing, at rates that vary between species, to come close to osmotic equilibrium with any natural or experimental variations in concentration of the external medium. In all experiments, when the external medium was diluted the blood of Scutus showed a corresponding decrease in concentration; conversely, when the external medium was concentrated, the concentrations of the ions in the blood increased. After an equilibration time of approximately eight hours,

the osmotic pressure of the blood was close to that of the external medium; these results showed that Scutus is poikilosmotic over the range (75-115%) of sea water concentrations used in this study.

The degree of dilution and/or concentration of the medium which a poikilosmotic species can tolerate varies widely between species. Some animals, though poikilosmotic over a certain salinity range, are able at very low salinities to regulate the concentration of their blood so that it becomes hyperosmotic to their environment. There are two main groups of poikilosmotic animals:

1) Those which take up water (or lose water) until the body fluids are isotonic, or nearly isotonic, with the external medium. In this case the animals simply behave as osmometers, and swell or shrink depending on whether they are placed in hypotonic or hypertonic media. Examples of this type are Dendrostomum (Gross, 1954), Mytilus (Potts, 1954b; Remane and Schlieper, 1958; and others), Arenicola (Krogh, 1939).
2) Those which actively excrete salts to bring their body fluids into equilibrium with a hypotonic environment. The excretion of salts is usually preceded by an initial phase of osmotic water uptake, which is evident by swelling and weight increase of the animals. Typical examples are the polychaete Nereis diversicolor (Beadle, 1937; Ellis, 1937) and the marine opisthobranch Aplysia (Bethe, 1934; van Weel, 1957).

Results from experiments in the present work show that Scutus loses salts when placed in hypotonic media. This is indicated by:

(a) weight loss, after the initial weight gain, when animals are placed in dilute media,

(b) the return of the animal to a weight somewhat less than its initial weight in normal sea water, when it is returned to normal sea water after a period in a diluted medium and

(c) a decrease in cation concentration in the blood of an animal placed in a medium isotonic with sea water, but with a lower salt concentration than sea water (e.g. a solution of three parts sea water and one part isotonic sucrose solution).

This salt loss may not be an active excretion, but rather evidence of simple diffusion and also, to some extent, due to damage suffered by the cells on exposure to hypotonic solutions.

Winterstein (1916) investigated the effect of injury on cell wall permeability and found that cell damage led, not only to increased permeability to salts, but also to a relatively greater increase in permeability to water. In experiments on Arbacia eggs, using fertilization test as a criterion of injury, Lucké and McCutcheon (1932) consistently found that an increase in permeability to water accompanied injury due to hypotonic solutions. Many other workers have also shown that injury is accompanied by increased permeability to various substances within and outside the cell.

The fact that Scutus did not recover when returned to normal sea water, after extended periods in hypotonic solutions, shows that irreparable damage had occurred during the time in which the animal was in the dilute medium. Van Weel (1957) was of the opinion that results of experiments which led Bethe (1930) to consider Aplysia to be an osmoadjustor, may not have represented normal responses of the animal, but might have reflected definite pathological conditions.

When van Weel determined oxygen consumption of

Aplysia after the animal was transferred to 80% sea water he found an enormous increase in oxygen consumption. This he considered without doubt to be partly caused by the increased motility of the animal. (Increased activity upon placement of animals in a hypotonic medium was also noted with Scutus, and Gross (1957) observed that an adverse osmotic environment stimulated an animal to random movements, or escape movements.) After about thirty minutes in the dilute medium, when the abnormal movement of Aplysia had ceased, there was a rapid fall in oxygen consumption, which slowed down after an hour, but the oxygen consumption curve continued its downward trend during the entire duration of the experiment. This occurred even when animals were transferred to 100% sea water after a stay of 5.5 hours in diluted sea water. In van Weel's opinion, this indicated that Bethe was working with probably dying, or at least abnormal, animals. It is possible that many results of other experimenters, also investigating osmoregulation of other marine invertebrates in dilute media, reflect abnormal responses of those species.

Therefore, in this study, results obtained from animals which had been in hypotonic solutions for extended periods, are not considered to be physiologically significant, but are useful in making comparisons with those obtained by other workers for other species. For example, weight changes of Aplysia (Bethe, 1930) and Scutus in 75% sea water are compared in Figure 11, and in a solution of three parts sea water / one part isotonic sucrose solution in Figure 15. In 75% sea water both Scutus and Aplysia show a similar pattern of weight change, but the degree of change of body weight of Scutus is not as great as that shown for Aplysia by Bethe. However, no conclusions about relative permeabilities of the body

surfaces of these two species can be drawn, since Bethe gave no indication of the sizes of the animals used in his experiments. Also, in this study it was shown that, for Scutus, animals of a similar body size did not necessarily show a similar increase in body weight when placed in the same hypotonic medium (Figs. 10 & 12). In the sea water/sucrose solution, Scutus like Aplysia, shows a loss in body weight. The large sucrose molecules are not able to diffuse into the animal as quickly as the cations diffuse out; this decreases the osmotic pressure of the blood and so water is lost from the animal, bringing about the observed loss in body weight. The body weight of Scutus increases when the animal is returned to normal sea water. As would be expected if salts were lost from the body during the time in which the animals were in the sea water/sucrose solution, Scutus, when replaced in normal sea water, does not return to its initial body weight, but to a weight somewhat less than that. In contrast, Aplysia, when returned to normal sea water, showed an increase in weight to 110% of its initial value in 100% sea water, and Bethe assumed that this increased weight was brought about because the skin is not quite impermeable to cane sugar. Florey (1966) cites Aplysia as an example of an animal which adapts to hypotonic solutions by excreting salt.

2.4.3 Relative permeability of the body wall to various ions

The permeability of the body surface depends, not only on the permeability of the membranes of the cells which make up the body wall, but also on the permeability of the intercellular cement, which binds cells together. The cell membrane is considered to be composed of a

TABLE 5

Diameters of the hydrated ions of the main inorganic ions in biological fluids. Data taken from Potts and Parry (1964, p.13)

Ion	Diameter Å (hydrated)
Na^+	5.6
K^+	3.8
Ca^{2+}	9.6
Mg^{2+}	10.8
Cl^-	3.8
SO_4^{2-}	7.2

bimolecular lipid layer with polypeptide chains of protein molecules adhering to both lipid-aqueous interfaces; in order to explain certain permeability properties, the presence of water-filled pores in the membrane has been postulated (see de Robertis, Nowinski and Saez, 1965). Both the cell walls and the intercellular cement are stabilised and made less permeable by the presence of calcium and, to a lesser extent, magnesium. Calcium is thought to be bound to the negative phosphoric sites of the phospholipids such as lecithin and cephalin (Tobias, 1958; Kimizuka & Koepsu, 1962). Alexander, Teorell and Aborg (1939) suggested that calcium binding to cephalin molecules made a tight molecular structure and thus reduced permeability. Martin (1953) pointed out that calcium binding regulated the degree of coiling of chondroitin-sulphuric acid, which is a sulphated mucopolysaccharide forming part of the structure of connective tissue. He suggested that calcium might thus affect permeability by altering the pore size of a structure.

Water and ions pass through a cell wall more quickly than would be expected if they passed through simply by diffusion in solution in the lipid layer, and it is probable that they pass through pores and/or in combination with lipid-soluble carriers within the membrane. If ions do pass through pores in the membrane, it would be expected that the rate of penetration would be inversely porportional to the diameters of the hydrated ions (Table 5). Solomon and co-workers (Solomon, 1960) calculated that, for the membrane of the human red blood corpuscle, the equivalent pore diameter was 7-8.4 Å, and Solomon stated that it might very well be that pores of that approximate size were characteristic of membrane architecture. Pores of that diameter would allow free

passage to fully hydrated sodium and potassium ions, but not to fully hydrated calcium and magnesium ions. However, Lindeman & Solomon (1962) found that the equivalent pore radius of the luminal face of intestinal mucosal cells, measured in experiments in which lipid-insoluble non-electrolytes were used, appeared to be 4.0Å.

Dakin and Edmonds (1931) showed that Onchidium was less permeable to magnesium and sulphate ions, than to sodium and chloride ions. Koizumi (1935) was able to show that, regarding rate of penetration through the integument of the holothurian Caudina, ions could be arranged as follows:

$$K^+ > Na^+ > Ca^{2+} > Mg^{2+}$$

and $$Cl^- > SO_4^{2-}$$

Webb (1940) and Conway (1956, 1960) have also obtained experimental evidence for faster penetration of cell membranes by small ions than by large ones.

When Scutus was subjected to changing external salinities such as were found at the Heathcote-Avon Estuary, the relative rates of change of concentrations of the blood cations were as follows:

$$Ca^{2+} > K^+ > Na^+ > Mg^{2+}$$

The relative rates of change of potassium, sodium and magnesium concentrations are those which would be expected if the degree of permeability of the integument to a cation and, therefore, the rate of loss of the cation from the body, is inversely proportional to the ionic diameter of the hydrated ion; however, the rate of decrease in calcium concentration is proportionately greater. Also during the initial period in which animals were in 75% and 85% sea water a marked decrease in blood calcium was evident.

Differences in rates of change of concentrations of individual ions in the blood of animals, which had been exposed to hypotonic solutions for more than an hour, were not taken to be physiologically significant. These conditions were more severe than those which would be experienced in the natural environment, and it was found that animals could not be maintained for extended periods in these dilute solutions. Therefore, under these conditions the animals were dying, and the cell membranes would have lost their normal characteristics of differential permeability to various ions. Not only would the change in blood concentration then be affected by the permeability of the external integument, but also, as the animal died, cell contents would have escaped from the dying cells and so would have altered the ionic composition of the blood.

Animals in a hypertonic medium showed blood calcium levels which were relatively higher than those of the other cations in the blood. The changes in calcium concentrations in the blood of animals exposed to hypo- and hypertonic media cannot be explained simply on the basis of diffusion of ions through pores in the membrane between the blood and the external medium.

As no studies were made to trace the actual movement of cations (for instance, by using radioisotopes) when animals were placed in the experimental media, it is impossible to come to firm conclusions concerning the observed changes in the concentration of calcium in the blood. However, it is of interest to note that Collip (1920) found, when he exposed the lamellibranch Mya arenaria to the atmosphere, that the calcium concentration of the coelomic fluid showed a considerable increase whereas, under the same conditions, the increase

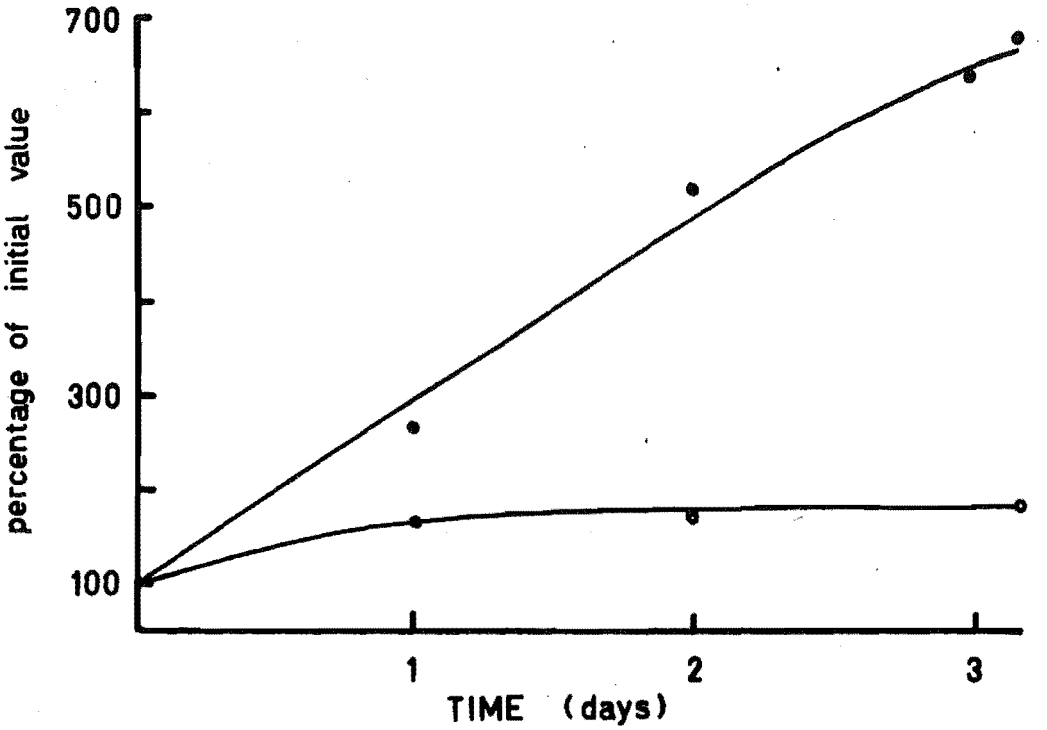

Figure 24: Increases in the concentrations of calcium (●——●) and magnesium (o——o) ions in the coelomic fluid of Mya when exposed to air. (data from Collip, 1920.)

in magnesium was slight (Fig. 24). The increase in calcium was found to be more or less parallel with the increase in combined carbon dioxide. A similar increase in calcium in the body fluids, when the pH is lowered by the accumulation of carbon dioxide, is shown in many animals with calcium-containing shells. In this case the shell is an important source of base for buffering (Prosser and Brown, 1961).

The facts that calcium is known to be taken up by the body fluids from the shell under certain conditions in other animals, and that it is known to be reversibly bound by cephalin and protein molecules which make up the cell membrane structure, indicate that a change could be expected in the concentration of calcium ions, different from that found for the other cations, which are not present in large concentrations in the shell and are not bound to the same extent.

When Scutus is placed in a hypotonic solution, free calcium ions in the body fluids may combine with molecules of the cell membranes, so reducing the permeability of the cell wall. Such binding of the calcium ions may explain in part the initially rapid decrease in calcium concentration of the blood when the medium surrounding the animal is diluted (Figs. 18 and 19).

It was observed that, when Scutus was placed in hypotonic solutions, there was a tendency for the animal to lose its black pigmentation. Gross (1951) found that calcium ions had a lytic action on the pigment granules of the Arbacia egg. An increase in the concentration of calcium in the body wall of Scutus might accelerate lysis of the pigment granules of an animal in a hypotonic solution.

2.4.4 Effect of temperature on the changes of concentrations of ions in the blood

Temperature is known to affect the ability of some species to withstand low salinities. Panikkar (1940) considered that, in euryhaline species where mechanisms for maintaining hypertonicity were well-developed, the range of tolerance to dilution of the environment was greater at higher temperatures than at lower ones. On this basis he stated that a marine species, which might have only feeble powers of regulation, could penetrate into brackish waters more easily at higher than at lower temperatures. Panikkar was familiar with species in tropical waters, and was apparently unaware that some species, e.g. Rhithropanopeus harrisi (Kinne and Rotthauwe, 1952; Verwey, 1957) and Gammarus duebeni (Kinne, 1952) can withstand low salinities better at low than at high temperatures. It would seem that his statement is only valid for species which have a greater osmoregulatory capacity at high than at low temperatures, e.g. Crangon crangon* (Broekema 1941; Verwey, 1957).

Verwey drew attention to the fact that osmotic pressure is a function of both concentration and temperature. Broekema had found that if Crangon was kept under conditions of constant salinity the blood concentration rose as the temperature fell. Verwey conducted some experiments on the osmotic behaviour of Crangon and found that, for a particular temperature, there was an optimal salinity for maintaining Crangon in

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* However, Flügel (1959) obtained results which contrasted with those of Broekema and Verwey; he found Crangon crangon to be a species which regulated more successfully at low than at high temperatures.

an aquarium; the higher the temperature, the lower was the optimal salinity. However, differences in osmotic pressure between the blood and the medium at the optimal salinities for the temperatures of 4°C and 21°C were the same. In the shrimps Penaeus aztecus and P. duorarum, Williams (1960) found an impaired capacity for osmoregulation at lowered temperatures of about 8.8°C. His results do not support Verwey's suggestion concerning the constancy of pressure differences.

Smith (1955), in work on Nereis diversicolor came to the conclusion that, in his experiments, temperatures of 7°, 14° and 20-21°C gave no significant differences in the concentrations of ions which were measured in the blood of animals exposed to media of different salinities.

Todd (1964b) found that temperatures of 5-15°C did not affect survival and blood concentration of Littorina littoralis in 100% and 75% sea water. However, low temperature favoured survival in 25% sea water. For the species L. littorea and L. saxatilis she found that above 50% sea water the temperature had no significant effect on the osmotic concentration of the blood.

From the results of other workers, quoted above, it can be seen that the effect of temperature on osmoregulation and survival of different species in hypotonic solutions varies considerably between species. The experiments conducted on Scutus do not show whether the tolerance of this species to dilution of the environment would be greater at high or at low temperatures. Temperature may not be so critical in affecting the animal's ability to withstand a hypotonic medium, as in its effect on the transport of oxygen by the blood. The oxygen affinity of the respiratory protein is decreased by an increase in temperature, while the metabolic rate and

hence the need for oxygen is increased by an increase in temperature (Scheer, 1963).

2.5 SUMMARY

1) Scutus breviculus, like most other marine invertebrates, is poikilosmotic (over at least the range, 115% to 75% sea water, which was investigated in this study), but is not able to survive extended periods in dilute media.

2) Exposure of Scutus to hypotonic media for short periods brought about an increase in body weight and a decrease in the osmotic pressure and cation concentrations of the blood; all these values returned to normal when the animal was returned to 100% sea water.

3) When Scutus was subjected to 85% or 75% sea water for extended periods, the condition of the animal deteriorated and resulted in death. Visible signs of physical deterioration were a decrease in muscle tone and a loss of surface pigmentation.

4) In a hypertonic medium (115% sea water) Scutus decreased in body weight, and the osmotic pressure and the cation concentration of the blood increased.

5) The rate of change in blood concentration of animals placed in hypotonic, or hypertonic, media was inversely related to the size and, therefore, to the surface area of the animal.

6) In experiments simulating environmental conditions, where dilutions were within the physiological range of the animal, the order of rate of decrease in concentration of the four cations measured in the blood was

$$Ca^{2+} > K^{+} > Na^{+} > Mg^{2+}$$

7) No significant differences in rates of change of blood concentration were found between animals kept at 7°C and those kept at room temperature.

SECTION 3

GROSS AND MICROANATOMY OF THE NERVOUS SYSTEM

3.1 INTRODUCTION

Before commencing electrophysiological studies of the nervous system, it was necessary to study the anatomical arrangement of at least those parts which were to be used in the physiological experiments. This included an investigation of the gross anatomy, using dissection and preparation of serial sections of whole specimens, and histological studies of certain parts of the nervous system.

Little cytological work has been done on the nervous systems of prosobranchs and, initially, histological examination of the nervous system of Scutus was made to determine the distribution of nerve cell bodies, fibres, glial cells, etc., in the ganglia and in the nerve cords of the central nervous system. However, some recent reports have given evidence of neurosecretory involvement in osmoregulation in some gastropods (Boddingius, 1960; Gorf, 1961; Lever, Jansen & Vlieger, 1961; Vicente, 1963) and it was decided to extend the microanatomical study of Scutus to see whether neurosecretory cells could be found in this species and, if there was some indication of neurosecretory material, to see if the amount of this material varied when animals were placed in media of different salinities.

3.2 METHODS

3.2.1 Study of the gross anatomy

(a) Dissection

After narcotisation in a medium containing equal parts of sea water and a saturated solution of magnesium chloride or sulphate, animals were either dissected under a binocular microscope immediately, or preserved and dissected later. Formalin, 70% alcohol, or a mixture recommended by Crofts (1929) for Haliotis*, were used for preservation. Dissections were made both from the dorsal and from the ventral sides.

The staining method of Hasbrouck (1959), using gold chloride and picric acid-iodine, was used to try and trace fine nerves, but it was not found to be very satisfactory. Examination of serial sections showed the distribution of fine nerves more clearly.

(b) Serial sections

Juvenile specimens, about one centimetre in length, were narcotised and then fixed in Bouin's fluid. They were cleared in terpineol and embedded in paraffin wax, using a vacuum embedding oven to ensure complete penetration. Serial longitudinal and transverse sections, 8μ in thickness, were prepared. The sections were stained with either Delafield's haematoxylin and eosin, or Weigert's iron haematoxylin and orange G (a saturated solution in absolute alcohol), or paraldehyde-fuchsin and Halmi's mixture (see method (b) below). These three staining methods were found to give good differentiation of cells, tissues and organs, and from a study of the sections stained by these different methods a

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* Croft's mixture for preservation: equal parts of 2.5% formaldehyde in sea water, 90% alcohol and glycerine.

clear picture of the internal anatomy of the animal was obtained.

3.2.2 Microanatomy and histology

Ganglia, commissures and connectives were dissected out from fresh specimens and fixed immediately. The tissues were cleared in terpineol, embedded in paraffin wax and sections of thickness 5μ or 6μ were prepared. A variety of fixatives and staining procedures was used to try to distinguish cell inclusions which might be neurosecretory material. The phenomenon of neurosecretion was first recognised in the hypothalamus of vertebrates and it was found that certain staining methods selectively stained the neurosecretory material. These same methods (e.g. the paraldehyde-fuchsin (1950) and chrome-haematoxylin (1941) methods of Gomori), or modifications of them, have been used to investigate neurosecretory phenomena in invertebrates. However, as Bern and Hagadorn (1965, p. 354) note, "There is no specific stain for neurosecretory material per se." Neurosecretory material in different groups of animals can consist of different chemical compounds, and therefore show different staining specificity. Bern and Hagadorn stated that protein, especially sulphur-rich protein, is the ubiquitous component, but carbohydrate and lipid constituents have been frequently reported. There is also evidence that the stain taken up by a structure is dependent on purely physical factors, particularly the density of texture which affects the ease with which stains of different diffusion coefficients are able to penetrate (Gabe, 1966).

Staining reactions alone provide an inadequate basis for conclusions regarding the occurrence of neurosecretory material. However, in this work, it was hoped that, by

using several staining methods that have been used to identify neurosecretory material in other animals, it would be possible to differentiate between the various inclusions in the neurons, and to see if the numbers or quantity of any of these inclusions showed any correlation with the dilution of the medium in which an animal had been kept prior to dissection. (An animal was not narcotised before dissection in this micro-anatomical study.) A change in the quantity of presumed secretory material with some physiologic condition would indicate the occurrence of a secretory cycle, and render the existence of neurosecretion probable.

Gabe (1966) wrote that the neurosecretory cells in prosobranchs were generally approximately the same size as the ordinary non-secretory neurons, their nuclei presented no particular features, but the features of the cytoplasm made identification easy. He stated that the secretory product had an affinity for azocarmine and iron haematoxylin. However, Andrews (pers. comm.) found that, when using Heidenhain's "Azan" staining method, the "inactive" neurons storing the neurosecretory substance stained with azocarmine, while the actively secreting ones, and the droplets along the axons, had a strong affinity for aniline blue. She found that both of these appeared white in living tissue and stained with paraldehyde-fuchsin. The behaviour of neurosecretory material towards paraldehyde-fuchsin varies in different species. Gabe considered that in all the Diotocardia the material, despite oxidation with permanganate, remained acidophilic and stained with the acid stains used after paraldehyde-fuchsin; however, Boddingius (1960), in a study of Patella, regarded positive staining with paraldehyde-fuchsin as an

indication of neurosecretory material.

After consideration of reports of staining affinities of neurosecretory material in other animals, particularly Diotocardian prosobranchs, the stains selected for use in this study were iron haematoxylin, paraldehyde-fuchsin with acid counterstains,* azocarmine with aniline blue in the counterstain (Heidenhain's "Azan" method) and stains specific for S-S groups (Alcian blue and an iron-resorcin lake of crystal violet, following oxidation with performic acid). The various procedures which were used are listed below:

(a) Fixative: Bouin's fluid
Stain: Weigert's iron haematoxylin
Counterstain: orange G, a saturated solution in absolute alcohol.

(b) Fixative: Bouin's fluid, with the acetic acid replaced with 0.5% trichloroacetic acid.
Stain: paraldehyde-fuchsin, prepared and used as recommended by Cameron and Steele (1959)
Counterstain: light green SF yellowish, orange G, chromotrope 2R (with phosphotungstic acid - see Halmi, 1952)

Before staining, sections were oxidised in Gomori's fluid (1941), rinsed in 2.5% sodium bisulphite and washed thoroughly with water. (If traces of bisulphite are left, the paraldehyde-fuchsin loses its staining specificity.)

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* Chrome-haematoxylin is often used for the investigation of neurosecretory phenomena, but it appears to have a staining specificity almost identical with that of paraldehyde-fuchsin, and so only P-F was used in this study.

(c) Fixatives: Bouin's fluid, formaldehyde-saline, or Carnoy

Stain: Azocarmine - 0.05% solution in distilled water, with glacial acetic acid added to bring the solution to 0.5% acetic acid.

Counterstain: aniline blue (0.15%), orange G (0.5%), and acetic acid (2%) in distilled water.

This method is a modication of Heidenhain's "Azan" staining method. The steps used in the staining procedure were the same as those recommended for the Heidenhain method by Lillie (1965), except that all staining was carried out at room temperature and the lengths of time for which the slides were left in the staining and mordanting solutions were shortened considerably.

(d) Fixative: formaldehyde-saline

Stain: Alcian Blue 8GS

The method used was that of Adams and Sloper (1956), in which sections were oxidised with performic acid (Pearse, 1953), before staining.

(e) Fixative: formaldehyde-saline

Stain: a mixture containing crystal violet, dextrin, resorcin and fuchsin (Humberstone, as cited by Dogra and Tandan, 1964)

As in the Alcian blue method, sections were oxidised with performic acid before they were stained.

3.3 GROSS ANATOMY

The gross anatomy of the nervous system of Scutus has been described by a few authors but, as there are discrepancies in the descriptions and as the terminology applied to the ganglia has not been uniform, a short

description of the central nervous system is included here.

The nervous system of Scutus, like that of other fissurellids (e.g. Fissurella (Boutan, 1885; Ziegenhorn and Thiem, 1926), Lucapina (Illingworth, 1902), Glyphis (Ziegenhorn and Thiem, 1926)) shows many features that suggest that the Fissurellidae, along with other families (Pleurotomariidae, Haliotidae and Scissurellidae) of the superfamily Zeugobranchia, are amongst the most primitive of living Gastropoda. Included in these primitive characters are:

(a) the pedal ganglia are elongated into long cords, which are linked by many transverse commissures;
(b) the pleural and pedal ganglia are closely associated, and linked to the cerebral by long connectives - a hypoathroid arrangement;
(c) the supra- and subintestinal ganglia are not very distinct from the visceral loop;
(d) most of the connectives are long and they often have superficial nerve cells;
(e) the branchial and pleural ganglia of the same side are connected indirectly by anastomoses between peripheral nerves arising from these centres - i.e., there is dialyneury.

The gross anatomy of the central nervous system of Scutus appears to be very like the arrangement of the nervous systems of other fissurellids (Fissurella, Lucapina, Glyphis), which have been described fully (see references cited above). It is rather similar to that of Haliotis, of which Crofts (1929) made a very detailed study. The description of the fissurellid nervous system given by Simroth (1896-1907) includes notes on Parmophorus (=Scutus) obtained from reports by

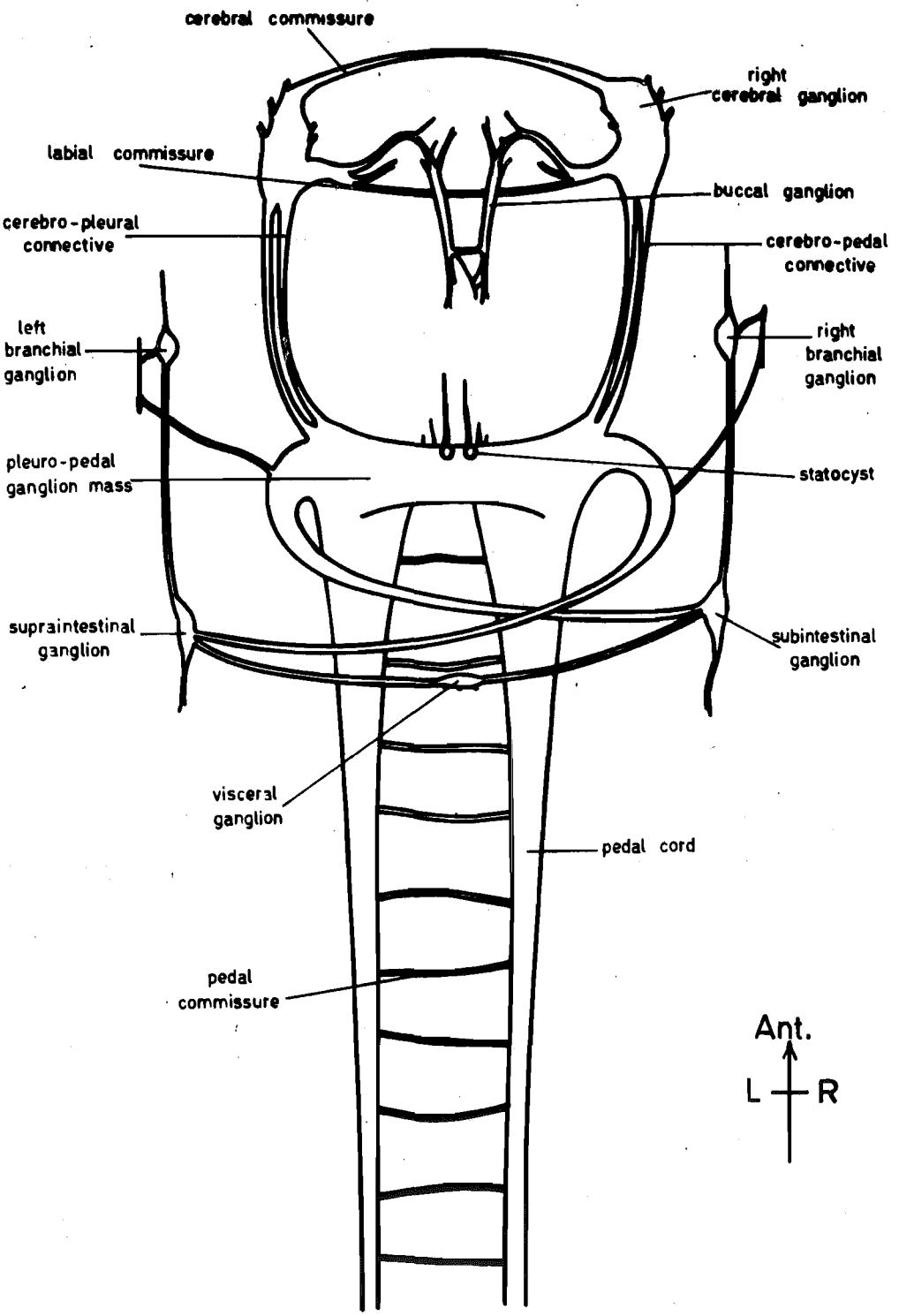


Figure 25: Diagrammatic representation of the central nervous system of Scutus.

the French investigators Boutan (1884, 1890a, 1890b) and Bouvier (1887). Boutan's work was done on specimens of an Australian species, at that time designated Parmophorus australis, collected at Sydney in 1880. His study is comprehensive but, unless the Australian species has an arrangement of ganglia markedly different from Scutus breviculus, contains some inaccurate observations. Boutan denied the existence of supra- and subintestinal ganglia whereas they are definitely present in Scutus, even though they are not very distinct. Bouvier supported Boutan's assertion that the supra- and subintestinal ganglia could not be recognised. This error may have been because their studies were made on preserved specimens only, in which the ganglia might not be noticeably differentiated from the nerve cords. In fresh animals the ganglia can be easily recognised, because the nerve cell bodies contain pigments (melanin, and an orange-yellow pigment - probably carotenoid - see section on cytology) which give the ganglia a definite orange-brown colouring, contrasting clearly with the white nerve cords. Risbec (1937) mentioned that the supra- and subintestinal ganglia of the New Caledonian Scutus unguis (=S. granulatus) were only slightly developed.

Tobler (1902) included a short description of the nervous system in his paper on the anatomy of Parmophorus intermedius; specimens for his study were collected in New Zealand, and were undoubtably the species now called Scutus breviculus.

Description of the gross anatomy of the nervous system of Scutus

A diagrammatic representation of the plan of the central nervous system is shown in Figure 25. (The

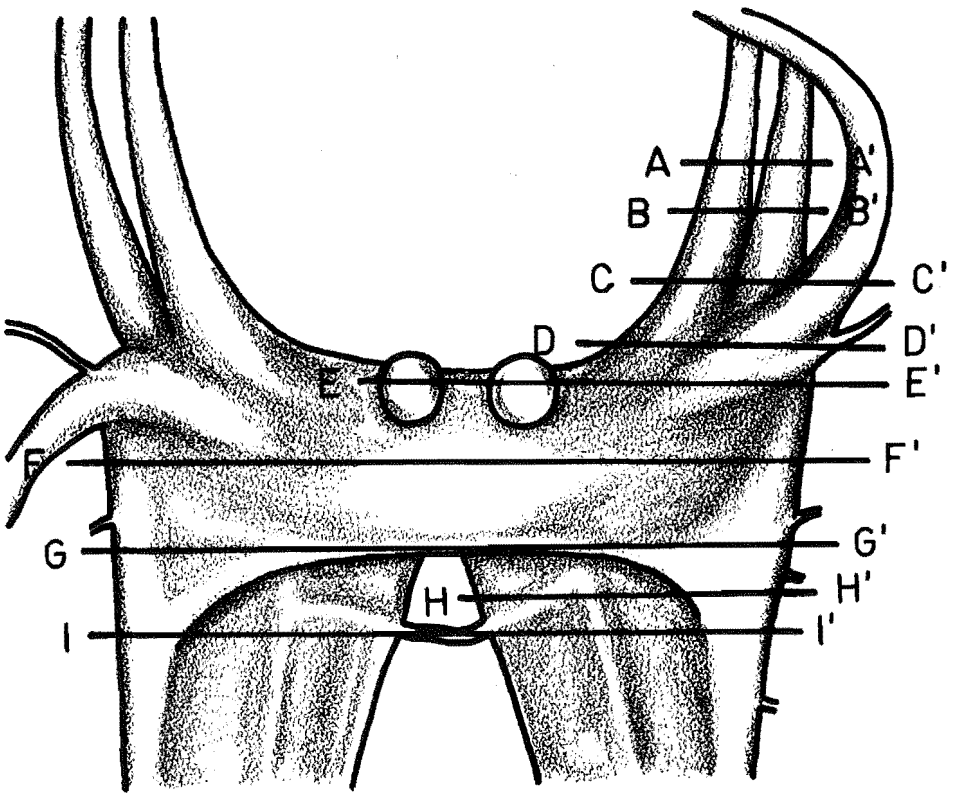


Figure 26: Dorsal view of the pleuro-pedal ganglion mass and associated nerves. Planes of sections in Figure 27 (a-i) are indicated by lines A-A' to I-I'. For names of nerves see Figure 25.

nomenclature used is that given by Bullock and Horridge (1965, p. 1293)).

1) The pair of cerebral (suboesophageal) ganglia are flattened and somewhat triangular in shape and lie laterally at the anterior end of the buccal mass. They are joined to one another by a long cerebral commissure. Anteriorly and laterally, nerves leave the ganglia to innervate the area around the mouth, the tentacles and the eyes. Posteriorly, a cerebropleural and a cerebropedal connective leave each ganglion, and the ventral part of the ganglion becomes extended into a gangliose projection (the "saillie labiale" of the French workers). This projection gives rise to two main nerves - (a) one which divides into two branches, one of which innervates the muscles on the floor of the buccal cavity, the second joining with the corresponding nerve from the opposite side to form a very fine labial commissure; (b) a cerebrobuccal connective which, after providing several branch nerves that extend into the buccal mass, leads to

2) the buccal (stomatogastric) ganglia. The two buccal ganglia are long and are joined by a posterior commissure to form a horseshoe-shaped nerve mass on top of the radular sheath, just under the beginning of the oesophagus. The commissure itself contains some superficial nerve cells and, for that reason, the whole horseshoe nerve structure has been considered to be ganglionic by some workers.

3) The pleuro-pedal ganglion mass is situated on the floor of the body cavity beneath the oesophagus. A series of transverse sections through the cerebropleural and cerebropedal connectives, the pleuro-pedal ganglion mass and the pedal cords is shown in Fig. 27. Fig. 26

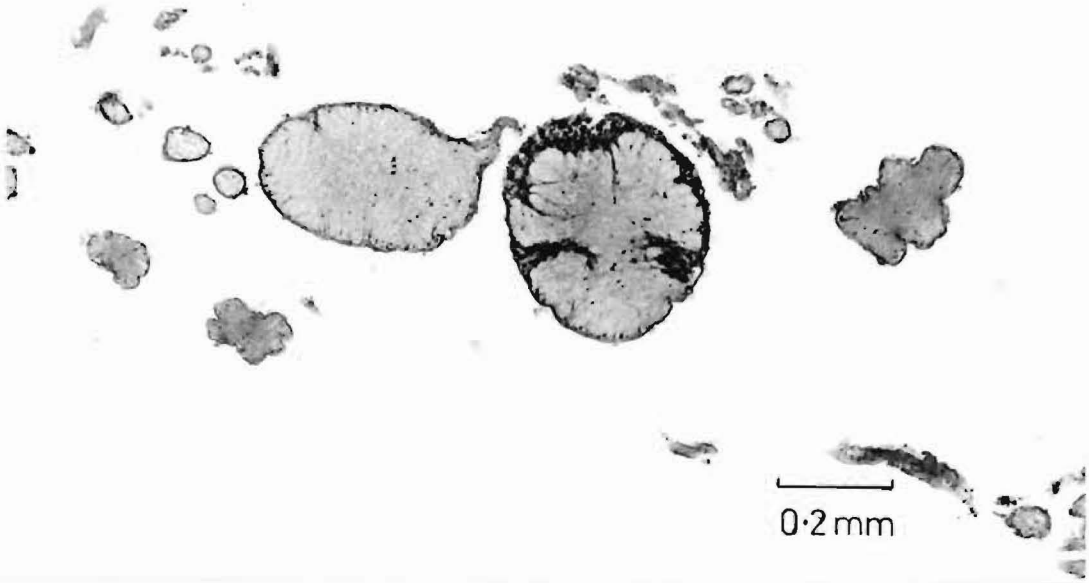


Figure 27a: Section at A-A' in Fig. 26. Right cerebropleural connective on left and right cerebropedal connective on right.

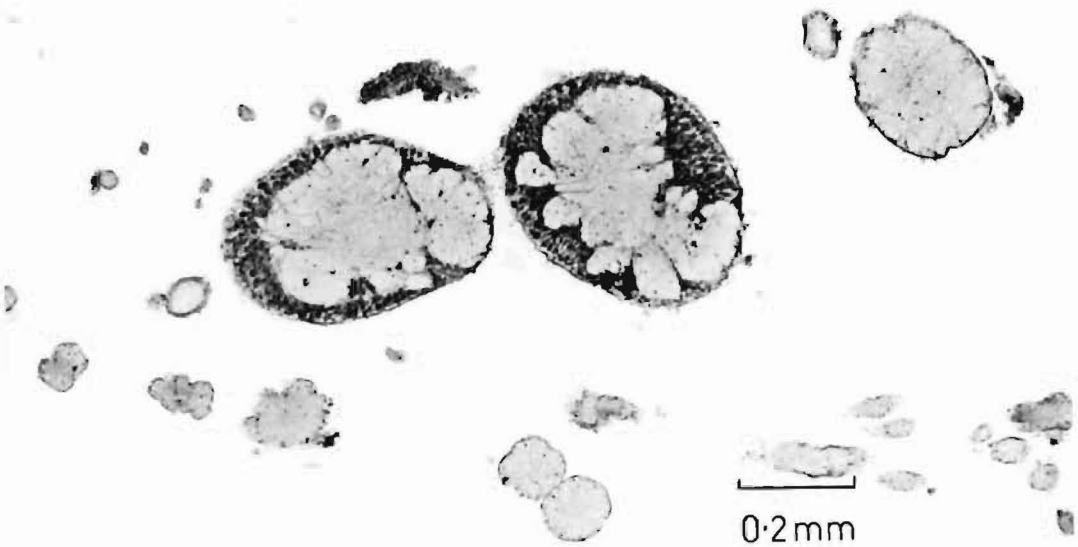


Figure 27b: Section at B-B' in Fig. 26.

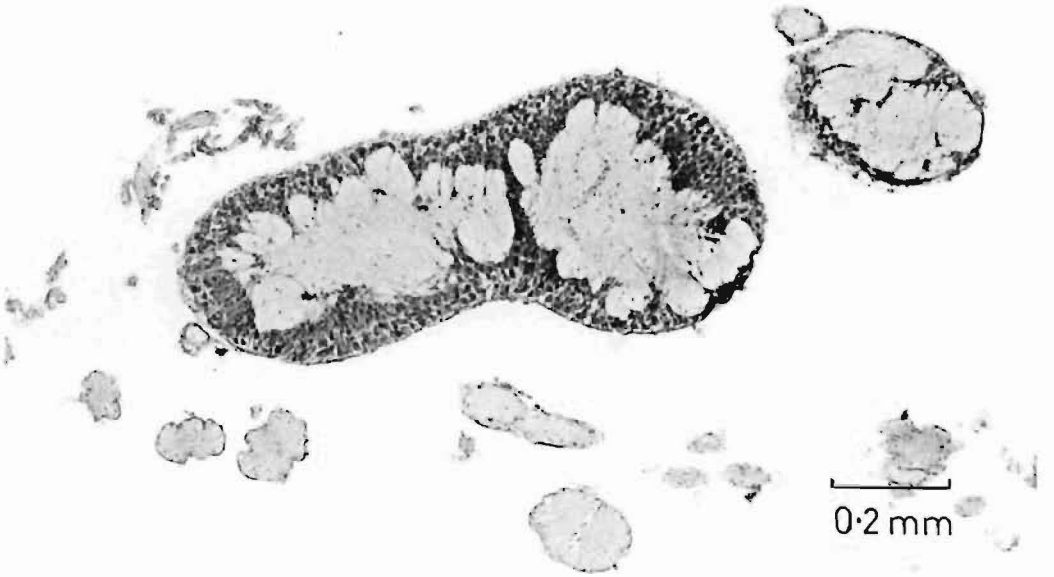


Figure 27c: Section at C-C' in Fig. 26. Cerebropleural and cerebropedal connectives joined. Pleural-supraintestinal connective on right.

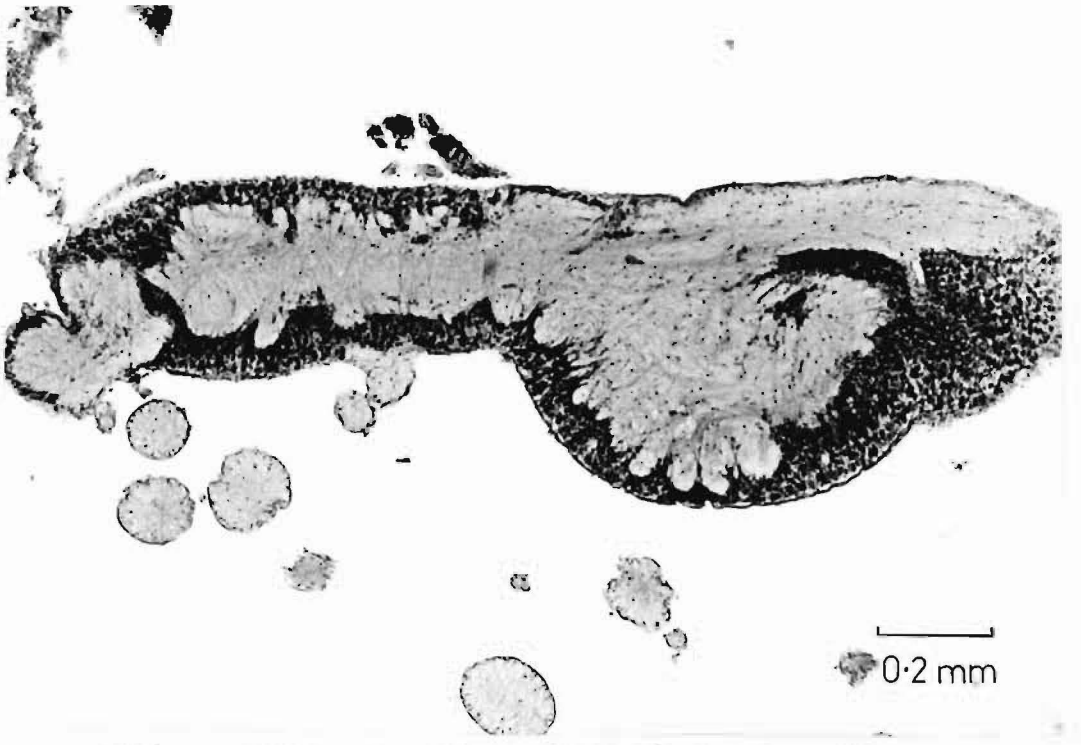


Figure 27d: Section at D-D' in Fig. 26.

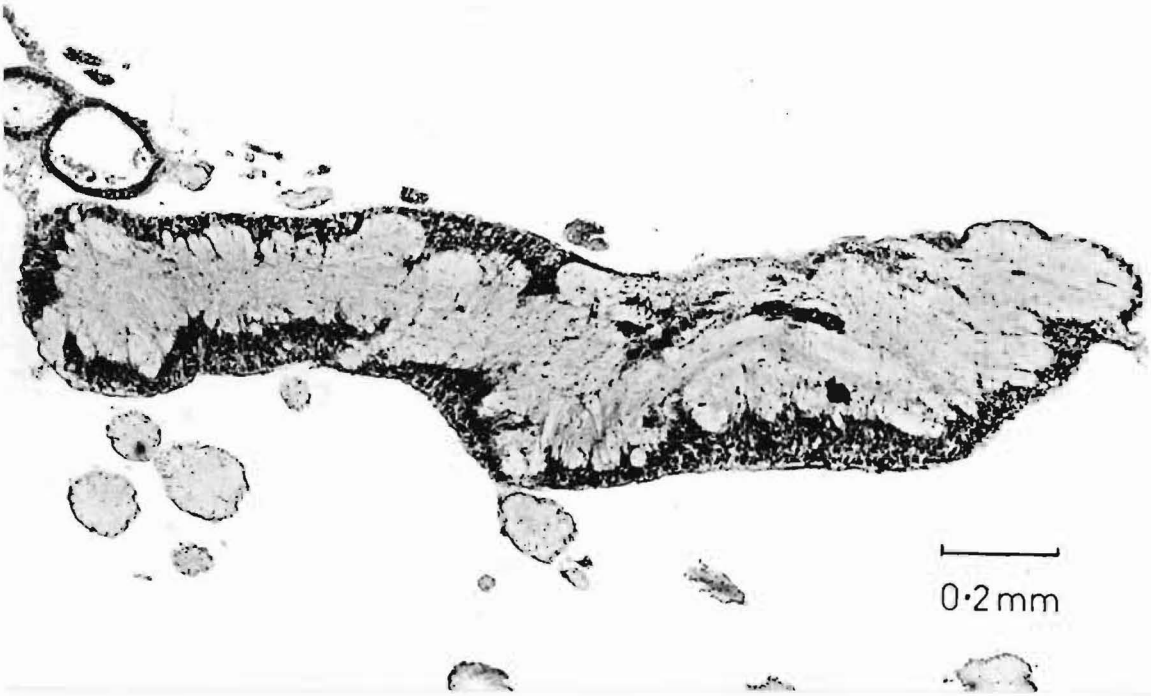


Figure 27e: Section at E-E' in Fig. 26. Statocysts visible in top left corner.

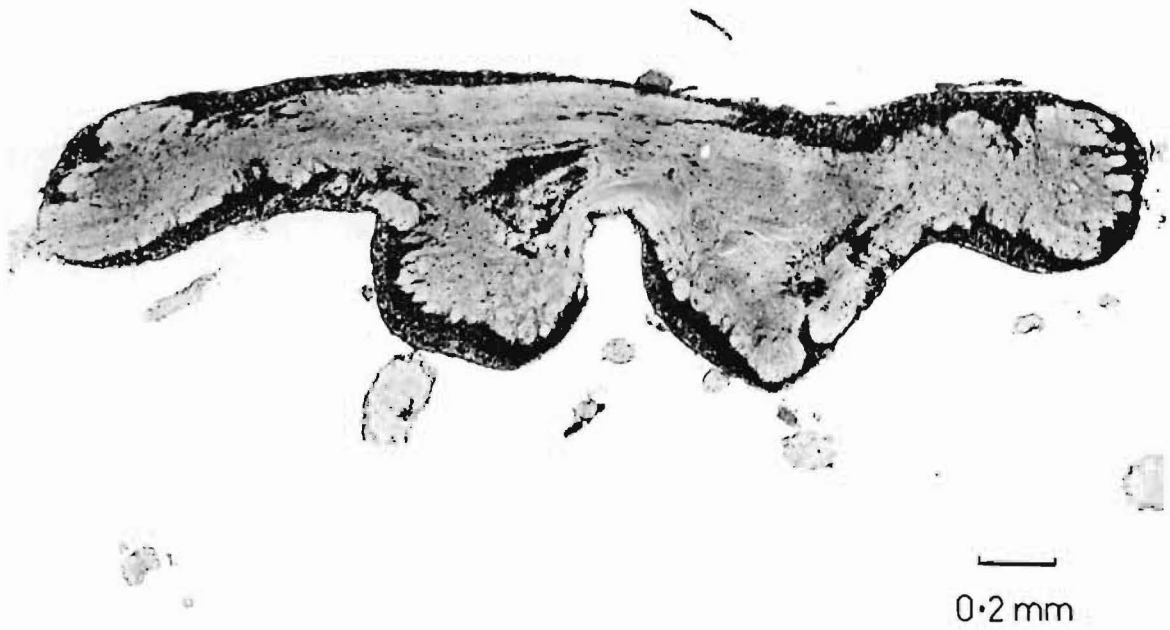


Figure 27f: Section at F-F' in Fig. 26. The pleuro-pedal ganglion mass.

Figure 27g: Section at G-G' in Fig. 26.

Figure 27h: Section at H-H' in Fig. 26. Right pedal nerve cord with portion of left pedal cord shown on left of photograph.

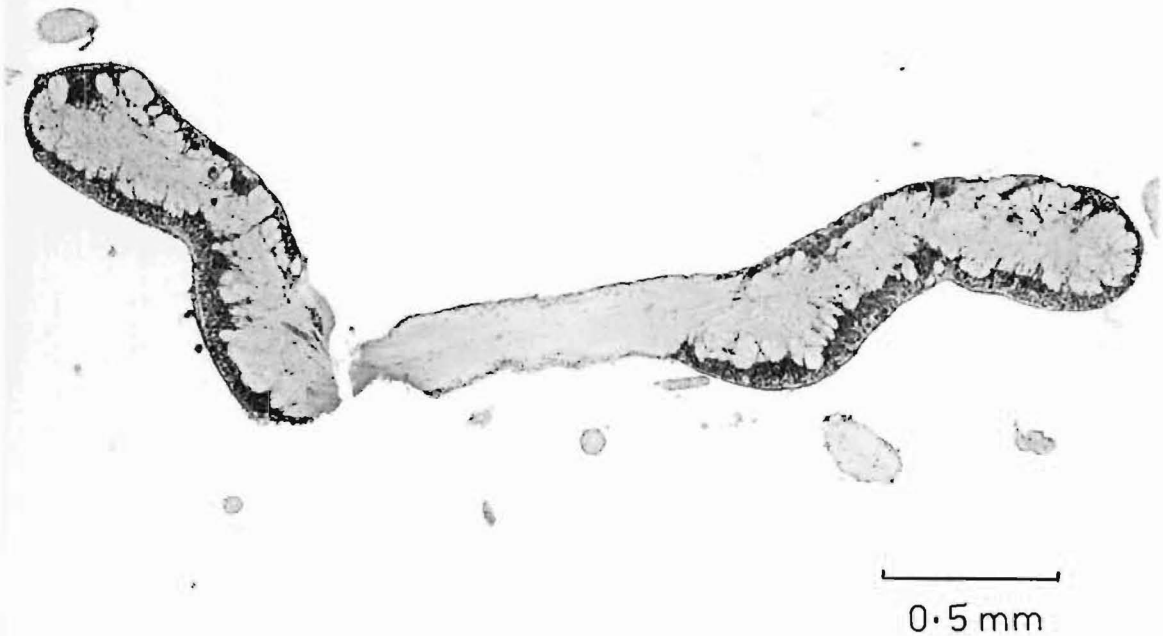


Figure 27i: Section at I-I' in Figure 26. Pedal nerve cords and pedal commissure.

shows the planes of these sections. The pleuro-pedal ganglion mass consists of

(a) a pair of pleural ganglia, situated just dorsal to the anterior end of the pedal cords, so that superficially the two cannot be distinguished. In sections (e.g. Fig. 27f) fibres can be seen to run transversely between the two pleural ganglia, and would appear to function as a commissure between the two ganglia. However, Bullock and Horridge state that the pleural ganglia of gastropods are not directly joined by a commissure; they further state that fibres are believed to pass through the pleuropedal connective and pedal commissure to the opposite ganglion, without interruption. The pleurals receive connectives from the cerebral ganglia and from the visceral loop, and the nerve cell bodies extend some distance from the ganglia into these cords (Fig. 27b). A pair of statocysts is situated on the anterior dorsal surface of the pleural ganglia (Fig. 27e).

(b) the pedal ganglia are extended as pedal cords in a blood sinus along the top of the sole of the foot and, just in front of the posterior end of the body cavity, they enter the muscle of the foot. Throughout their length they are very folded, allowing extension when the foot is extended. Even when the muscles of the foot are extremely relaxed after narcotisation, the pedal cords are not fully extended. These ganglia are connected by many commissures (one is shown in Fig. 27i), which give a ladder-like appearance. In cross section each cord is oval, with a lateral depression (Fig. 27h). This lateral groove has led some investigators, including Boutan and Bouvier, to consider that the upper portion of the ganglion cords was pleural in nature and the lower part pedal, the groove marking the division between the

two. Other workers opposed this view (see Ziegenhorn and Thiem, 1926, for a review of the subject). Crofts (1929) observed in Haliotis that the lateral grooves of the pedal cords were ventral to the grooves marking the division between the pleural and pedal ganglia at the posterior end of the pleuro-pedal ganglion mass, and not continuous with them, as de Lacaze-Duthiers (1887) had supposed, when he initially described two distinct nerves in one 'neurolemma'.

Although the pedal cords of Scutus are very similar in form to those of Haliotis, the position of these cords in relation to the musculature of the sole of the foot is quite different. In Haliotis they penetrate into the foot muscle just posterior to the pleuro-pedal ganglion mass and, therefore, throughout their length they are embedded in the muscle. In contrast to this arrangement, in Scutus, as has been mentioned, the pedal cords are surrounded by a blood sinus lying on top of the muscle, and they penetrate the muscle only at the posterior end of the body cavity.

The pedal ganglia innervate the foot, epipodium and mantle. The various nerves which innervate the mantle anastomose to form a nerve ring, which contains many cell bodies, and may be regarded as a diffuse peripheral ganglion cord.

4) The intestinal ganglia - have been called parietal ganglia by some workers, but Bullock and Horridge consider that this latter term should be restricted to those ganglia found in the Opisthobranchia and Pulmonata which are thought to be a detached portion of the pleural ganglia. In Scutus, they comprise:

(a) the supraintestinal ganglion, which is situated adjacent to the body wall on the left dorsal side of the

body, slightly posterior to the pleuro-pedal ganglion mass. This ganglion is connected to the right pleural ganglion by a nerve (part of the visceral loop) crossing the body dorsal to the gut. Two other connectives lead from the supraintestinal ganglion, the first anteriorly through the muscle to the left branchial ganglion, and the second transversely to the visceral ganglion.

(b) the subintestinal ganglion, which lies against the body wall on the right side of the body cavity, in a more posterior and ventral position than the supra-intestinal ganglion. Connectives lead from this ganglion to the left pleural ganglion (the nerve crossing ventral to the gut and between it and the pleuro-pedal ganglion mass), the right branchial ganglion and the visceral ganglion.

Each of the intestinal ganglia gives off a fairly large nerve, which travels posteriorly and which, in Haliotis, Crofts calls the internal pallial nerve.

5) The visceral ganglion. This ganglion is small and only appears as a slight swelling on the visceral loop. It lies just over the rectum, near the kidney duct. It supplies nerves to the posterior part of the gut, the heart and the kidney.

6) The branchial ganglia are well-developed and are situated laterally to the ctenidia, near the branchial veins, about a third of the way along the ctenidium from its base. Each branchial ganglion has a connective to the intestinal ganglion of the same side. Laterally there is a fine nerve, which leads to the pallial nerve ring and this forms an indirect connection uniting the branchial and the pleural ganglia of the same side.

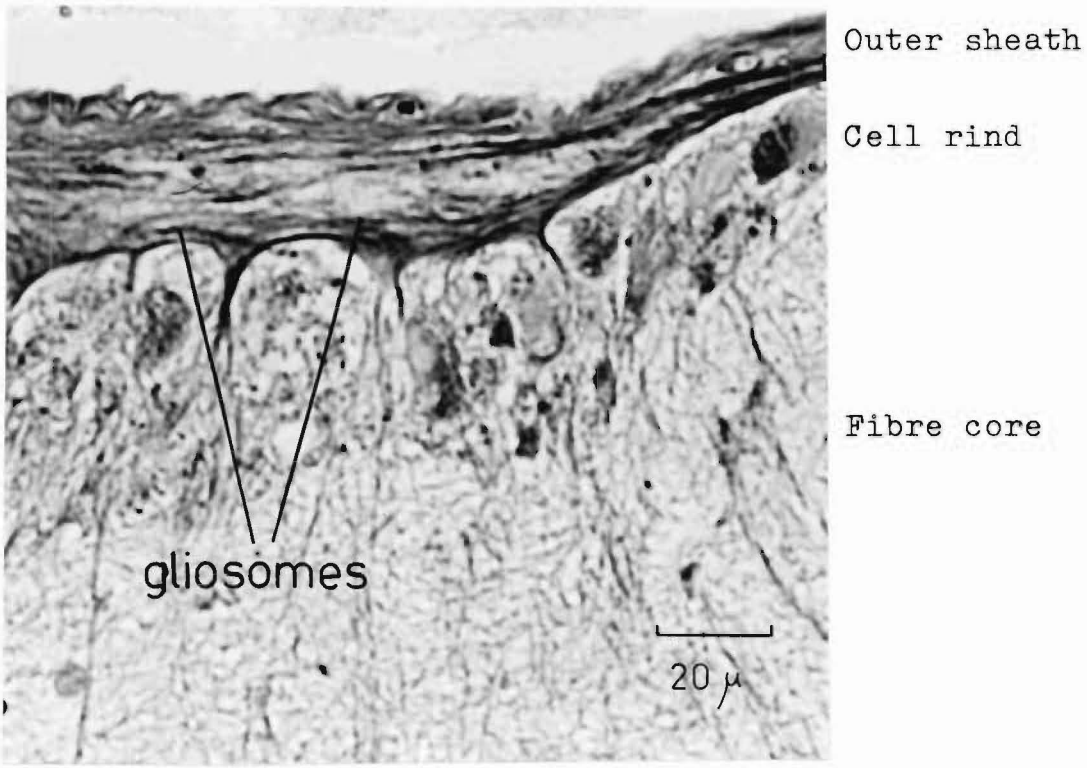


Figure 28: Section of a cerebral ganglion showing sheath, with extensions of the glial cells of the perilemma between the cells of the cell rind layer. P-F stained material can be seen in some of these processes. (Section 5μ - stained with paraldehyde-fuchsin and Halmi's mixture, method (b), p. 66.)

3.4 MICROANATOMY

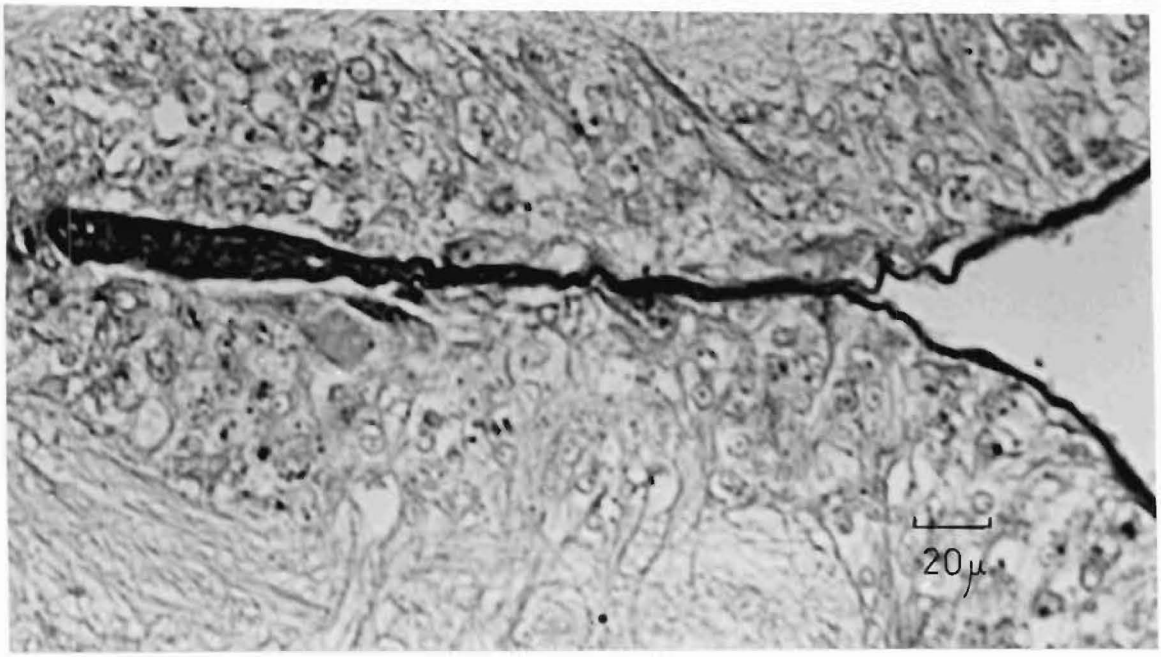
3.4.1 Ganglia

Each ganglion consists essentially of an outer sheath, a layer of cells (the cell rind) and, inside this, a fibrous core (Fig. 28).

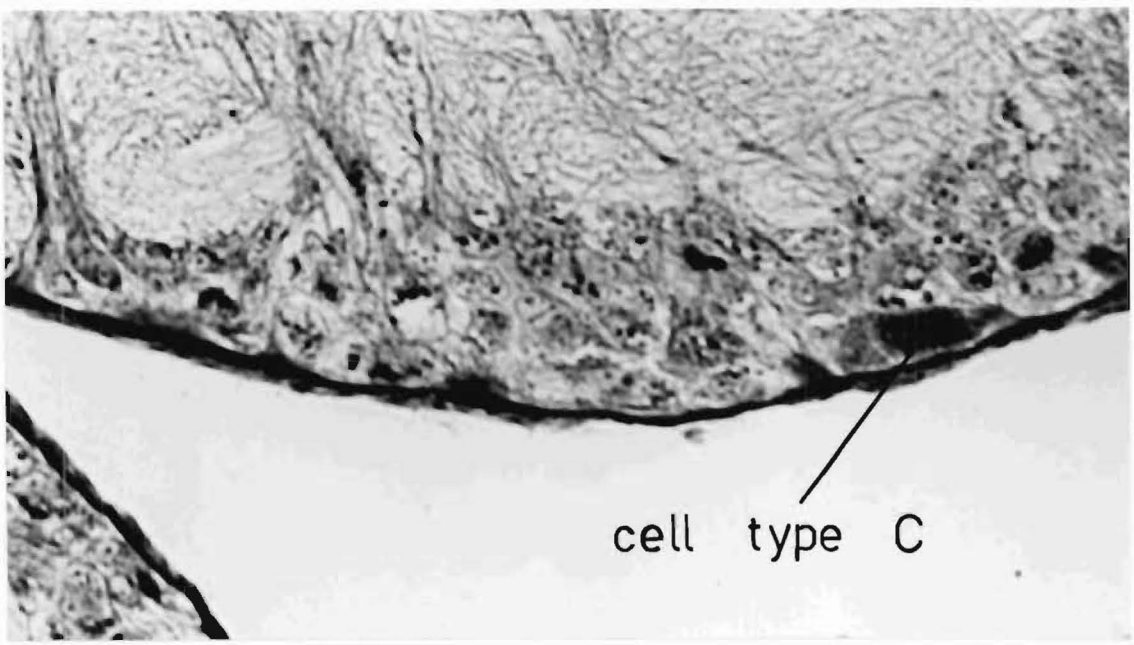
(a) The outer sheath

This sheath is usually about 4μ thick, although in parts of the cerebral ganglia, especially in concave regions, it may be as great as 35μ. It consists of an outer fibrous connective tissue layer (the neurilemma) and an inner layer (the perilemma), formed of many neuroglial cells. (Some workers have considered the neurilemma to be secreted by the underlying perilemma cells.) These two layers of the sheath are clearly differentiated by some of the staining methods used in this study. For example, in sections stained with paraldehyde-fuchsin and counterstained with Halmi's mixture, the perilemma stained positively with paraldehyde-fuchsin, while the neurilemma took up the light green stain strongly. Light green is a good stain for collagen fibres (Carleton and Drury, 1957) and in the Crustacea and Insecta, also, the neurilemma has been shown to stain like collagen (Bullock and Horridge, 1965).

Sometimes the material in the glial cells which took up the paraldehyde-fuchsin stain appeared as discrete granules, which probably correspond to the gliosomes (=pigment bodies), which have been described in the glial cells of other invertebrates. Pipa (1961) identified such granules in Periplaneta, and histochemical data accumulated by him indicated that the gliosomes consisted of glyco - or phospholipids probably closely associated with protein. For Aplysia, Rosenbluth (1963) obtained



(a) Animal (♂) in 75% sea water for two hours prior to dissection.



(b) Animal (♂) in normal sea water prior to dissection.

Figure 30: Sections of pedal ganglia stained with paraldehyde-fuchsin and counterstained with Halmi's mixture - method (b) p. 66.

histochemical evidence of some glycogen particles in the glial cells, as well as the lipid and pigmented gliosomes. "Gliagrana" have been described in the perineurium of Crepidula by Nolte, Breucker and Kuhlmann (1965). Inclusions have also been found in the glial cells of Helix pomatia (Schlote, 1963; Nolte et al., 1965) and, in experiments on Helix, it was found that there was a variation in the number of these inclusions in the cells with the age, physiological condition, etc. of the animal. The decrease or increase in the granules in the glial cells was found to be correlated with changes in the number of similar paraldehyde-fuchsin-positive granules (= cytosomes - see section b (ii) p. 83) in the nerve cells.

In some regions, particularly in the pedal ganglia, the inner layer of glial cells penetrates between the cells of the cell rind, sometimes extending right into the fibre core (Fig. 28). These septa-like processes have been recognised in Unio tumidus by Fährmann (1961) and in Aplysia by Rosenbluth (1963).

(b) The cell rind

(i) Distribution of ganglion cells

The cell rind consists of a tightly packed layer of cells, several cells deep (Fig. 29). The thickness of the cell rind varies in different parts of a ganglion, and is interrupted where fibres and cords leave the ganglion (e.g. Fig. 27h). In some places nerve cells may penetrate quite a distance along a nerve cord, this diffuse nature of ganglia being most marked for the buccal ganglia and the ganglia of the visceral loop.

Almost all the cells are unipolar, with the axon usually directed towards the core of the ganglion. On

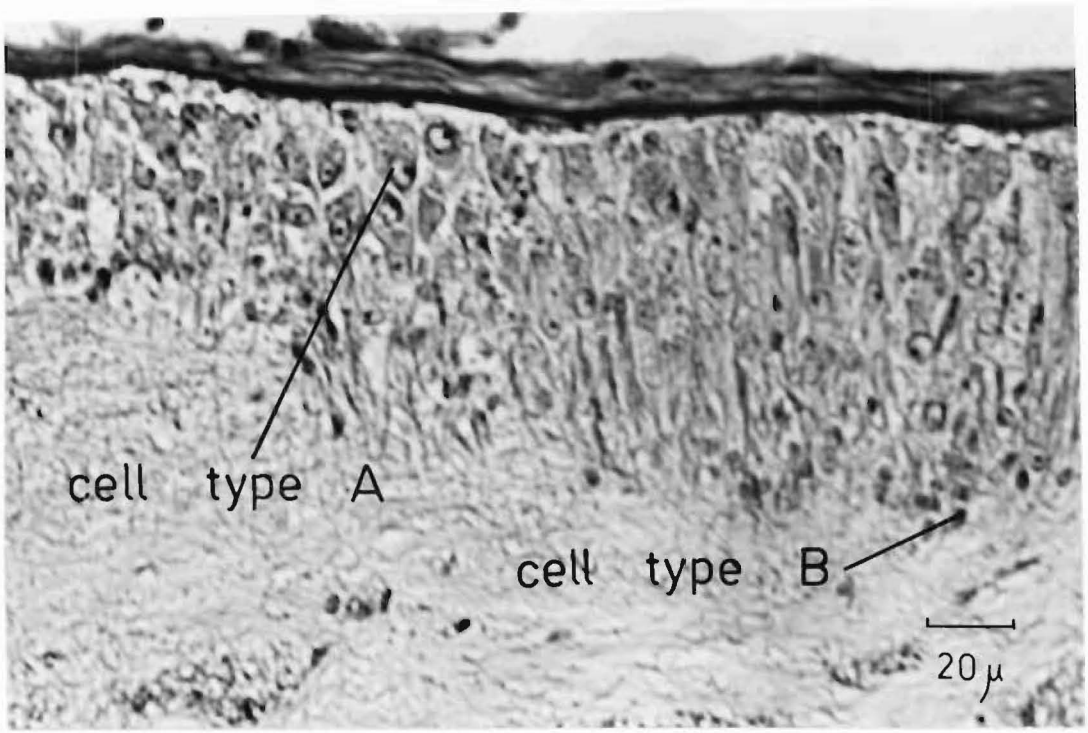


Figure 29: Section through a part of a cerebral ganglion, showing the ganglion sheath (top of photograph), the cell rind and the inner fibre core. (Section 5 µ - fixed in Bouin's and stained with a modification of Heidenhain's "Azan" stain, method (c), p. 67.)

the basis of size, shape and staining reactions, the cells can be divided into three main types (Figures 29 & 30b).

Cell type A

These are large cells (up to 40μ long) with abundant cytoplasm which usually shows granular inclusions. They have large, spherical nuclei (3-5μ) with little chromatin, and a single, distinct nucleolus, which stains strongly with orange G or azocarmine. These cells are found in all ganglia and are usually alongside cells of type B. However, in some regions, for example certain areas of the cerebral ganglia, they are found in groups, oriented in the same direction and with no cells of type B between them.

Cell type B

These cells are smaller (approx. 10μ in length) than those of type A; the nucleus is not regularly shaped and has high chromatin content, taking up both orange G and azocarmine very strongly. There is not as much cytoplasm as in cell type A.

Cell type C

This type of cell was observed in the pedal ganglia, and was most common in the ventral, median region of the pleuro-pedal ganglion mass, in the area where the two pedal cords were beginning to become differentiated from one another. These cells are large (usually about 20μ long, but they may be up to 45μ), oval or pear-shaped, and the cytoplasm is almost completely filled with 'yellow' globules (see p.80).

(ii) Cytoplasmic inclusions of ganglion cells

As Bullock and Horridge (1965, p. 81) point out,

any discussion about cytoplasmic inclusions must be preceded by notes of caution. Many of the common techniques of fixation and embedding remove much of the organic matter from the neurons. Even by the best techniques, it has been estimated (Hydén, 1960) that the nerve cell cytoplasm can lose 50% of its organic material and the nucleus 80%. Also, treatment with strong reagents is likely to produce artifacts.

The nature of the inclusions in the nerve cells of gastropods has been a matter of dispute for many years. Inclusions in gastropod nerve cells have been investigated using phase-contrast microscopy, a variety of staining techniques for observation by light microscopy and, recently, electron microscopy. This variety of methods of study has produced an equally varied number of terms to apply to the inclusions in the neurons. In some cases, structures described by one author are obviously the same as those given a different name by another author; e.g. lipochrome (Legendre, 1913), vacuoles and vacuomes (observed "apres coloration vitale au rouge neutre") (Parat, 1928), binary spheroids, 'mulberry' spheroids, Golgi spheroids, metaplasmic granules (Thomas, 1947, 1948), spheroid complexes (Cain, 1948), lipochrome globules (Moussa, 1950), lipochondria (Roque, 1954), neutral red or lipoidal bodies, (Lacy and Rogers, 1955; Lacy and Horne, 1956), spheroidal bodies (Lacy, 1957), lipid spheroids (Malhotra, 1957), lipid globules, 'yellow' and 'blue' globules (Chou, 1957a, 1957b; Chou and Meek, 1958), phospholipid and mixed lipid droplets (Meek and Lane, 1964, Lane, 1964), dense inclusion bodies (Baxter and Nisbet, 1963) and cytosomes (Nolte, Breucker and Kulmann, 1965) are, at least in part, synonymous. The term 'dictyosomes' (rods, batonets, crescents, rings) probably refers to the same structures after they have been treated with osmium or

silver techniques for staining Golgi apparatus. Nissl substance was described by Hydén (1960) as a substance, present in most cell bodies of vertebrates and invertebrates, which was easily stainable with basic aniline dyes, and which was in the form of large granules, thin plates, or in a more diffuse fine-granular form. He stated that, in fresh cells, it was readily stained with methylene blue, a phenomenon which has been noted by other authors for some of the inclusions mentioned above. Electron microscope studies have now identified the Nissl substance as consisting of the so-called endoplasmic reticulum and granules ('microsomes') 100-300 Å in diameter.

In recent years, even more confusion in the terminology of the inclusions of the neurons of gastropods has arisen out of studies investigating the occurrence of neurosecretory phenomena in this group. Neurosecretory material was first described in the Gastropoda by Scharrer in 1935. Gabe (1954, 1966) reported that neurosecretory cells were present in the Diotocardian (=Archaeogastropoda) molluscs, to which order Scutus belongs. However, Bern and Hagadorn (1965) considered that the presence of neurosecretion in the Diotocardia was still uncertain.

In some groups of invertebrates a paraldehyde-fuchsin method can be satisfactorily used for the identification of neurosecretory material but, in gastropods, other cell inclusions such as the pigment granules and the lipid inclusions mentioned above, stain positively with the Gomori paraldehyde-fuchsin method, or a

modification of it such as was used in this study.* Smith (1966) in a study of the central nervous system of the slug Arion ater, defined neurosecretion as "paraldehyde-fuchsin positive material found in the nerve cells and axons". He noted that neurosecretory material seemed to be present in varying quantities in all neurons of Arion ater at some time during the life cycle. Bern and Hagadorn considered that much of the so-called neurosecretary material claimed to exist in gastropod neurons might really be pigment of no secretory importance. From the abundance of neurosecretory cells and secretion granules described by Boddingius (1960) for Patella, and used by her as evidence of neurosecretory involvement in osmoregulation in that genus, it would appear that the positively-stained granules she observed were probably not 'neurosecretory', in the restricted sense of a neurohormone substance, but rather lipid and/or pigment substances. Gorf (1961) located secretory granules staining with paraldehyde-fuchsin in nerve cells of the cerebral, visceral and pedal ganglia, and of the connectives of the prosobranch Viviparus viviparus. He identified a group of five to ten especially large cells on each side of the cerebral ganglia and found that the secretory granules that took up the paraldehyde-fuchsin stain

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* Following oxidation, staining with paraldehyde-fuchsin is probably due to ionic links with the acidic product of cystine oxidation (Landing, Hall & West, 1956; Sumner, 1965), and staining without oxidation is probably non-ionic, although the chemical groups responsible have not been determined (Sumner, 1965). Goldstein (1962) considered that the positive reaction of P-F with elastic tissue might be hydrogen bonding.

might be scattered in the cell or thickly crowded. They were sometimes visible in the axon, giving the impression of migration along the axon. The granules were found to be wanting in juveniles and first appeared at an age of 54 days; they varied with season, temperature, light and salinity, and they increased during aestivation.

Some cytoplasmic inclusions can be identified even in fresh, unstained nerve cells of Scutus, so they are not just artifacts of fixation or staining procedures. The appearance of these inclusions agrees with that described by Chou (1957b) for Patella vulgata. Under the light microscope he observed two types of cytoplasmic inclusions, which he called 'yellow' and 'blue' lipid globules. The 'yellow' globules were so named because they contained a pigment which gave them a yellow appearance in unstained material; the 'blue' globules were found to have an affinity for methylene blue. (The same terms will be used in this work.) Electron microscope studies (Lacy and Rogers, 1955; Lacy and Horne, 1956; Lacy, 1957) have given a more detailed picture of these inclusions in Patella, and several studies have been made of similar inclusions in the snail Helix, and a few other gastropods (see Nolte et al., 1965, for a good review).

In Scutus, 'yellow' globules are clearly visible in cell types A and C. In cell type A they are usually accumulated at the opposite end of the cell from the nucleus; they are not quite spherical, and are about 1-2 μ in diameter. In cell type C they are present in large concentrations throughout the cytoplasm - in fact they often fill the cytoplasm so completely that no nucleus can be seen. The yellow colour in unfixed material may be due to the presence of

carotenoid, which has been shown to be present in gastropods (Cain, 1948; Chou, 1957a, 1957b). When cells are vitally stained with neutral red these 'yellow' globules take up the red stain internally, but the outermost layer of the globule does not stain. This staining reaction led Lacy and Horne to call these globules 'neutral red bodies'. For Helix, Chou (1957a) found that the 'yellow' globules were essentially lipid (cerebroside, phospholipid and carotenoid), but they also contained some tyrosine and histidine, and probably also some carbohydrate. It seems that these globules, which in this study were found to stain intensively with paraldehyde-fuchsin, would be the 'donkere secretiegranula' (dark-coloured secretion granules) described for Patella by Boddingius, which she noted decreased in number when the animal had been exposed to a hypotonic environment.*

Variation in the number of these globules in different animals was also noted in Scutus, but this variation could not be correlated with the salinity of the external medium. In the first series of sections prepared, it appeared that the numbers of paraldehyde-fuchsin-positive granules in the neurons were greater in the ganglia of animals kept in normal sea water, than in those of animals kept in diluted sea water for two hours (see Figures 30a & b). However, examination of many

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* In another investigation concerned with the effect of osmotic pressure on the inclusion in neurons, Young (1953) found that the neutral red globules in the neurons of cephalopods could be shrunken or swollen osmotically.

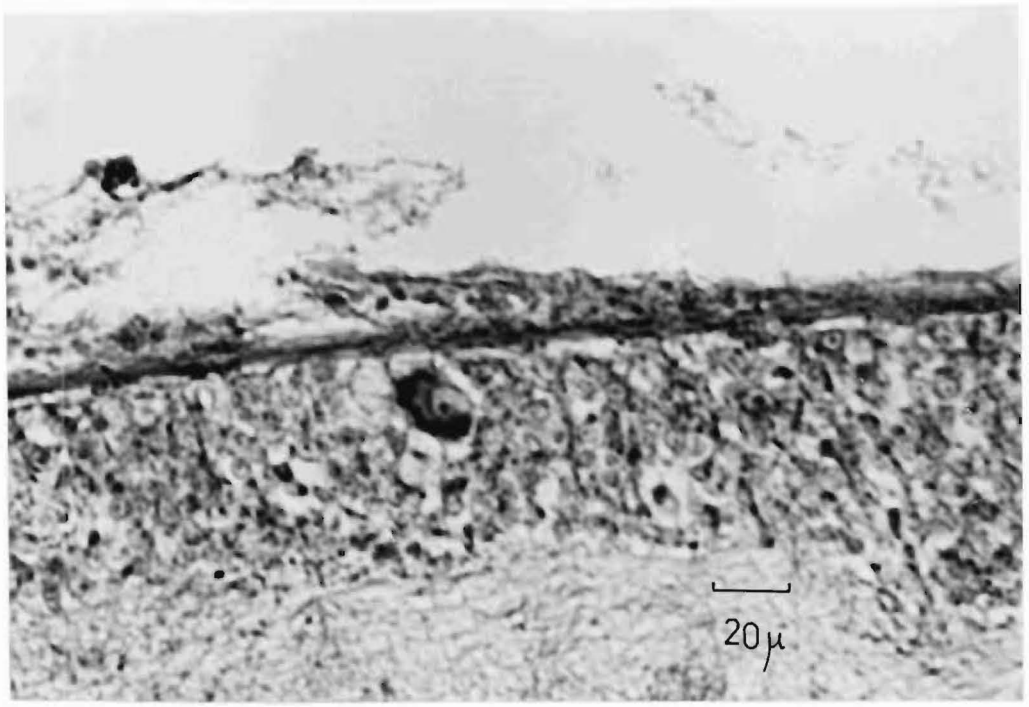

Figure 31: Large cell with P-F positive granules in the cerebral ganglion of an animal kept in 50% sea water for two hours prior to dissection. (Section 8μ - stained with paraldehyde-fuchsin and counterstained with Halmi's mixture, method (b) p. 66.)

more sections of ganglia of animals which had been kept in a variety of dilutions of sea water for differing lengths of time, revealed that such a variation in number of granules could be found in different animals kept in any one concentration of sea water. Therefore, although the external salinity might have some effect on these granules in the neurons, there must be other factors involved in determining the quantities of these granules. Even though, as Gabe (1966) has pointed out, the neurosecretory cells in primitive groups are not as concentrated into definite regions of certain ganglia as they are in more advanced species, the abundance of these granular cells in all the ganglia of Scutus would suggest that they are not specialised neurosecretory cells. Gabe stated that the very abundance of the 'neurosecretory cells' in gastropods, after staining by the methods that were classical for the hypothalamic neurosecretory product, should be enough to lead investigators to carry out control tests to determine if the stained substance in the neurones was not neurosecretory material but only pigment and lipid substance, which has been shown to stain with chrome haematoxylin and paraldehyde-fuchsin in the Gomori methods. In only one case in this study was it possible to differentiate any cell from those surrounding it, on the basis of form and staining affinity. A single large cell, quite distinct from smaller neighbouring cells, was seen in the cerebral ganglion of a specimen which had been kept in 50% sea water for two hours (Fig. 31). This cell had a large nucleus which was circular in section and an accumulation of paraldehyde-fuchsin stained granules around the periphery of the cytoplasm.

Small globules, like those described as 'blue' globules by Chou, and phospholipid droplets by Meek and Lane (1964), were found in large accumulations in the

central area of cell type A, between the nucleus and the 'yellow' globules. These globules were about 0.5μ in diameter, were spherical and colourless in unfixed material, but took up vital methylene blue. When stained by the paraldehyde-fuchsin-Halmi method, these globules stain positively with light green, and with the Azan method they are weakly stained with aniline blue. Chou and Meek (1958) considered the ultrafine structure of the globules of Helix suggested the 'yellow' globules originated from the 'blue' globules. In an electron-microscope study of the inclusions in the nerve cells of the giant snail Archachatina, Baxter and Nisbet (1963) identified two types of dense inclusion bodies, the smaller being about 100-400 mμ and less dense than the larger inclusions which were about 1μ in diameter. They thought it possible that the former were transformed into the latter.

Nolte et al. (1965) discussed experiments of their own and of others to establish the function of the transitory inclusions ("cytosomes") in gastropod neurons. They concluded that the inclusions were physiologically important metabolic products which, under some conditions where the energy requirements of the cells were low, were built up as a depot of reserve energy in the form of lipid and then, in other conditions which led to an increase in catabolic activity, they were decomposed. The 'yellow' and 'blue' globules of Scutus appear to belong to this class of cytosomes, and so variation in the amounts of these globules in the neurons of different animals would be expected.

Methods (d) and (e) (p. 67), which are specific for S-H groups, and which have been used by some workers to give selective staining of neurosecretory material,

did not stain any inclusions in the neurons of Scutus. (The only region of the nerve which was stained by these methods was the perilemma.) As has been noted previously, staining reactions of neurosecretory material are very variable, and a method which differentiates neurosecretory material in one group of animals may be completely unsatisfactory for another. Thus, the negative results obtained in this work do not indicate that neurosecretory phenomena are absent in Scutus but rather show that, if neurosecretory material is present, it is not in the form of a compound with free SH-groups, as is the neurosecretory substance of vertebrates and many other invertebrates.

In the living state, neurosecretory cells are characterised by a bluish-white opacity, which can be observed under a dissecting microscope when strong illumination is used. An examination of the nervous systems of freshly dissected specimens of Scutus did not reveal any cells with a bluish-white appearance, which suggests that neurosecretory cells, if present, are not large and prominent.

In mature animals many melanin-pigmented granules were observed in the cytoplasm of the neurons. When sections were oxidised with acid-permanganate, these granules became bleached and then took up the paraldehyde-fuchsin stain.

(c) The fibre core

Within the fibrous cores of the ganglia could be distinguished definite tracts of nerves fibres which extended into the nerves leaving the ganglia; intermingled with the fibres were neuroglia cells, whose nuclei were clearly visible.

3.4.2 Nerves

Like the ganglia, all the commissures, connectives and peripheral nerves*, have an outer sheath composed of neuroglia cells. Nuclei of neuroglia cells are also visible within the fibre tracts. As was mentioned earlier, the ganglion cells may extend along the nerve cords for some distance.

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* This histological study was made of the central nervous system, and so it is only the proximal parts of the peripheral nerves which were closely examined.

SECTION 4

NEUROPHYSIOLOGY

4.1 INTRODUCTION

A large amount of neurophysiological research has been carried out using the giant axons of cephalopods. However, information on the physiological properties of the nervous systems of gastropods, and other molluscs, is much more scarce; general neurophysiological studies have been made of the pulmonate gastropods Ariolimax columbianus (Turner and Nevius, 1951) and Archachatina (Calachatina) marginata (Nisbet, 1961; Baxter & Nisbet, 1963). The gastropod about which the greatest number of papers on neurophysiology has been published is the marine opisthobranch Aplysia. This animal has giant nerve cell bodies into which microelectrodes can readily be inserted, and the French workers Cardot, Arvanitaki-Chalazonitis, Chalazonitis, Fessard, Tauc, and others, have conducted experiments to investigate various properties of the nerve cells, especially rhythmic activity and synaptic transmission. Effects of temperature on the impulse frequency of the cells in the visceral ganglion of Aplysia have been studied by Murray (1966). Austin, Yai & Sato (1967) have examined the effects of calcium levels on the membrane potentials of the ganglion cells; they found that calcium had a marked effect on the excitability of the cell membrane, and also affected the permeability coefficients of at least water and sodium through the membrane.

The only information of ecological significance, which was found in the literature, on effects of concentration of the bathing medium on the activity in the

nervous system of gastropods, was from a few papers on osmoreception and activity of ganglia in some pulmonates. The activity of pulmonates is known to increase under normal conditions when the humidity of the environment is increased, and some investigations have been made to determine whether there are changes in the activity of the central nervous system (particularly the pedal ganglion) when it is subjected to different osmotic conditions. Hughes & Kerkut (1956) and Kerkut & Taylor (1956) studied the effect of changes in osmotic concentration of the medium on the frequency of spontaneous firing in the isolated pedal ganglion of the slugs Anjolimax reticulatus and Arion ater.* Dilution of the bathing medium was found to cause increased activity in the ganglion. They suggested that the change in haemolymph concentration brought about by a change in environmental humidity could be the cause of the increased activity of animals in moist conditions. Koshtoyants and Katalin (1961), from results of experiments on Helix pomatia, and Rózsa (1963), who studied Arion empiricorum, Limax maximus and Helix pomatia, disagreed with the explanation for change in activity which had been put forward by Hughes, Kerkut and Taylor, and were of the opinion that peripheral osmoreceptors present in the soles of the feet of these animals trans-

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*In the discussion of their paper, Hughes and Kerkut noted that persistence of electrical activity in parts of the central nervous system when isolated from the rest of the animal appeared to be a property of the nervous systems of all groups that had so far been studied. They pointed out that the interpretation of such activity and its significance in the behaviour of intact organisms presented problems at all levels of study in the attempt to bridge the gap between behavioural studies and neurological experiments.

mitted impulses to the nervous centres which, in turn, brought about increased activity.

Scutus usually does show increased activity when initially placed in a diluted medium, and changes in the spontaneous activity of the nervous system, similar to those found for pulmonates in the experiments mentioned above, may be present, but were not investigated in the present work. Instead, effects of dilute media on conduction of artificially induced impulses in the nerves were studied. If Scutus has been kept in diluted sea water for several hours, its movements become slower than normal, and an increase in activity upon transfer to a new medium (either more or less concentrated) is not as pronounced as when transfer is made from 100% sea water. Results of earlier experiments (Section 2) showed that when Scutus is kept in a dilute medium its blood is measurably diluted, and it was proposed to see if the 'sluggishness' observed in an animal in dilute sea water could, in part, be explained by effects of the diluted blood on the nervous system. The nerve membrane potential, spike height, conduction velocity, refractory period, transmission within the ganglia and at the nerve-muscle junction, etc. are all likely to be affected by changes in the osmotic pressure and ion concentration of the surrounding medium. However, for reasons given in Section 4.3 (b), conduction velocity alone was chosen as the parameter to measure.

In addition to experiments carried out to study the effects on the nervous system of Scutus of dilutions greater than those to which an animal would be exposed in its natural environment, some experiments were conducted to investigate the effects of dilutions actually

experienced in its estuarine habitat. These experiments investigated whether the minimum concentration of the blood, which could be expected in an animal, due to dilution of the sea water during a tidal cycle in the Heathcote-Avon Estuary, produced any measurable effect on the conduction velocity.

Some factors, which Bullock and Horridge (1965, p. 149) refer to as primary velocity correlates, are known to play a part in determining the velocity of spike propagation in a particular nerve. One of the obvious factors correlated with conduction velocity is the diameter of the nerve fibre. In the myelinated fibres of vertebrates, the velocity is directly proportional to the diameter (Hursh, 1939; Gasser and Grundfest, 1939), but it appears that for other animals and fibre types there are different velocity regressions, including some that approach a square root function (see, e.g., Pumphrey and Young, 1938). Very little is known of this factor for gastropods. A second important factor is the actual residual intrinsic nature of the membrane, reflected in its response to the rising intensity of the local current flow from preceding regions of activity. A third factor which can be correlated with conduction velocity in a general way is sheath thickness. As Bullock and Horridge point out, it probably is not mere physical thickness which is pertinent, but some other variable such as compactness x thickness, or lipid density, or ion permeability.

In addition to the above mentioned primary factors, some secondary factors are also important in influencing conduction velocity, and should be taken into account when measurements of conduction velocity are being made. Of the following secondary factors considered here, the

last four (nos. 4-7) were studied in some control experiments.

1) The frequency of the stimulating impulse. Bullock (1951) found that there was a small acceleration in the second and subsequent impulses conducted five or ten milliseconds apart in some species, but not in other species. In all experiments carried out here it was decided to stimulate at a constant frequency of one per second, during the period when measurements were being made. This stimulation frequency was found to be high enough to enable easy reading of the trace on the long-persistence C.R.O. tube face, and low enough not to cause fatigue in the nerve when it was stimulated repetitively.

2) Nerve fibres often taper or show irregular fluctuations in diameter, so that the absolute conduction velocity might vary in different parts of the fibre whose over-all velocity is being measured. This is one variable which could not be measured in the following experiments but, as direct comparisons of absolute conduction velocities were not to be attempted in the dilution experiments, this factor was not considered to be critical. The conduction velocities measured for different preparations (unstretched connectives at room temperature) in the initial experiments, studying the electrophysiological properties of the connectives (Table 7), were very similar. Therefore, it would seem that the conduction properties of the connective must be fairly uniform in different regions of the nerve, since the placement of the electrodes on the connective certainly varied from preparation to preparation.

3) Ionic composition of the medium around the fibre. This variable was investigated in the final experiments,

an endeavour being made to hold the other variables constant.

4) Time elapsing from dissection and initial recording, to making measurements. It is well-known that a nerve gradually loses its properties of excitability after its removal from the animal (see, e.g., Hodgkin, 1938).

5) The effect of stretch on the nerve.

6) The temperature of the nerve and of the bathing fluid.

7) The effect of mechanical disturbances to the nerve during dissection and during the course of the experiment.

4.2 METHODS

When this investigation was first planned it was intended that the pedal cords would be used for the electrophysiological experiments. These cords are very easy to dissect out from the animal, as they lie within a blood sinus on the top of the sole of the foot (see section 3.3). However, initial experiments showed that neurons in these ganglion cords exhibited a considerable amount of spontaneous activity, which tended to interfere with the measurement of artificially induced impulses. The cerebropedal and cerebropleural connectives are without cell bodies, except in regions close to the ganglia, and were found to give much more clearly defined action potentials than did the pedal cords. Therefore, the connectives were used in the experiments which are described in this section.

At the beginning of this work a considerable amount of time was spent in trying a variety of methods of stimulation and recording, and types of moist chambers, in order to determine the most satisfactory setup for the series of experiments which were to be conducted.

4.2.1 Apparatus

After a survey of the literature, it appeared that the most satisfactory electrodes to use for stimulating and recording would be suction electrodes. The anticipated advantages of such electrodes over the conventional external metal electrodes were that

(a) a shorter length of nerve would be required and

(b) the entire nerve would, at all times during the experiment, be immersed in the physiological solution and, therefore, the deleterious effects of air or oil would be eliminated. Also, it could be certain that the whole nerve would be exposed in the same way to a change in concentration of the surrounding medium.

Suction electrodes have been quite commonly used as stimulating electrodes, but they appear to have been seldom used as recording electrodes. Easton (1960) and Florey & Kriebel (1966) have both described suction electrode assemblies for stimulating and recording. They both reported that the action potentials recorded were as large as those obtained with external wire electrodes. A suction electrode assembly requires

(a) a suitable hydraulic suction system which will enable fine control of suction, and

(b) electrode holding-tubes with tip diameter only slightly larger than the nerve. This permits maximum suction and holding power, maximum height of action potential and minimum recorded stimulus artifact.

A suction-electrode assembly (Appendix III) was constructed and tested for this work but, in spite of the advantages of the suction electrodes already mentioned and of the fact that the nerves maintained

their activity for a long time under these experimental conditions, suction electrodes were not used to obtain the results shown in this work. The main problem experienced in recording from such an electrode system was that a large stimulus artifact was obtained, and this obscured the spikes of some of the fastest fibres in the connectives. An effort was made to reduce the stimulus artifacts but, even so, a system using conventional silver-silver chloride external electrodes for stimulation and recording was found to give records in which the compound action potential of the connective was more clearly defined from the stimulus artifact. Figures 32a & b show the arrangement which was used for recording from the nerves in the experiments outlined on the following pages. The nerve was held firmly in position by fixing the ends in draftmen's drawing pens (f_1, f_2), which acted as screw-controlled forceps, and the tension of the nerve was controlled by passing it under a fine glass hook (g). The position of this hook and other parts of the assembly could be adjusted by the use of Palmer D37 rack-work "X" blocks on a Palmer D2 stand. The distance between the stimulating and recording electrodes varied slightly from preparation to preparation, but was always within the range 1 - 1.5 cm. for unstretched nerves.

When using external electrodes for stimulating and recording, many investigators raise the nerve preparation into a layer of paraffin oil when measurements are being made, as the oil prevents drying of the nerve. Because of higher external resistance the conduction velocity is slower in oil than in sea water (Hodgkin, 1939). Katz (1947) obtained a much more marked effect of dilute solutions on reduction of conduction velocity

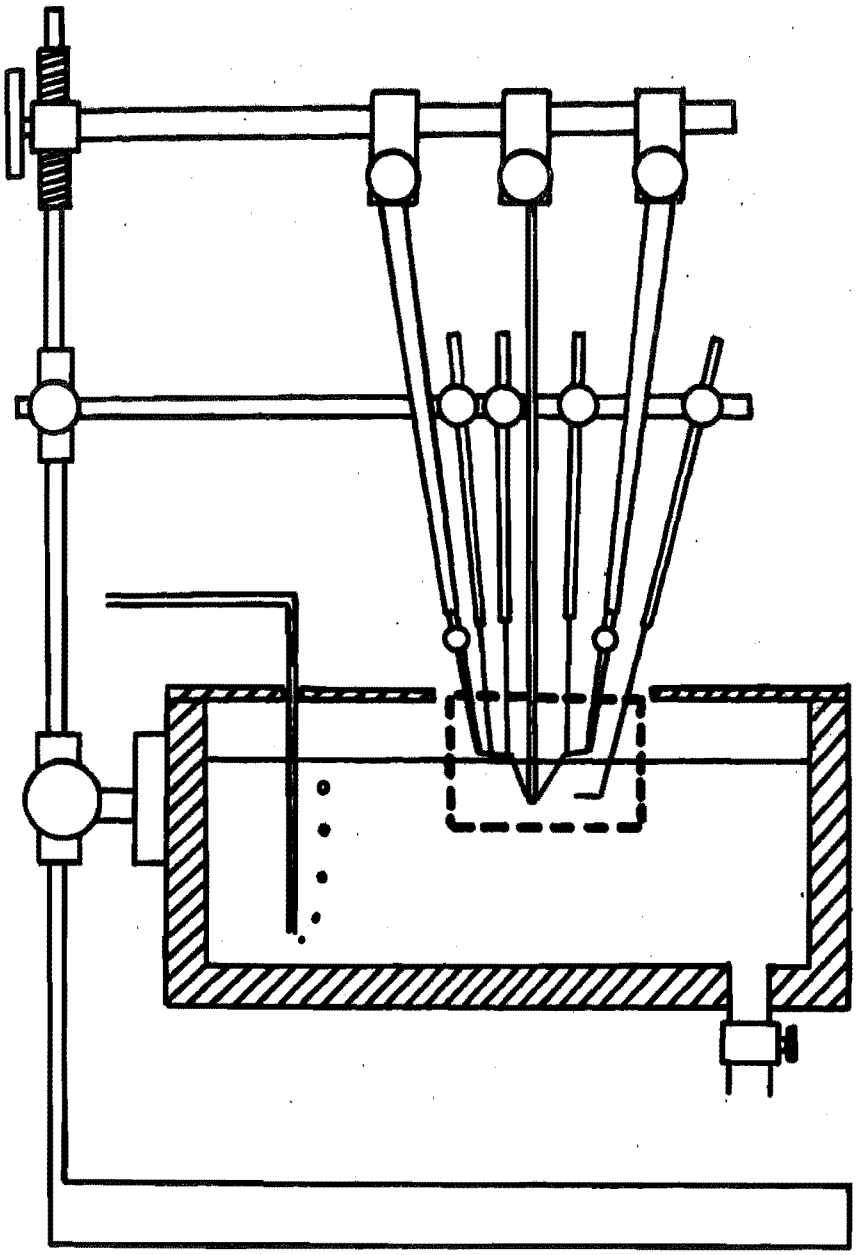
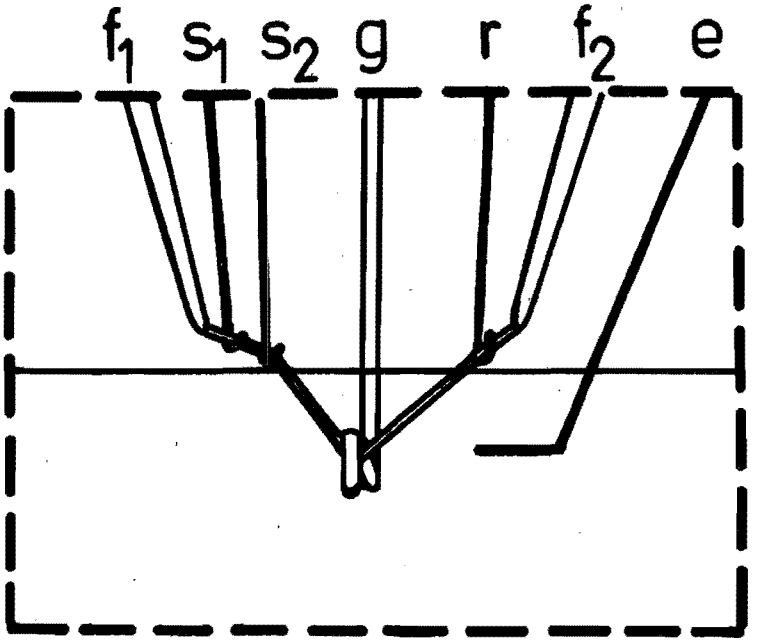


Figure 32a: Arrangement of nerve chamber and electrodes used in electrophysiological experiments.



s_1, s_2 - stimulating electrodes
r - recording electrode
e - earth electrode
f_1, f_2 - screw-controlled forceps
g - glass hook

Figure 32b: Detail of arrangement of nerve.

in a non-medullated nerve fibre of Carcinus maenas, when recordings were made with an axon in oil, than when recordings were made with the axon in a large bath of solution. For example, when the salt content of the surrounding fluid was reduced to one half of its original concentration, the conduction time was increased by 51% when the axon was in oil, and by only 4.77% when it was surrounded by a large volume of solution. In oil the nerve is surrounded by only a thin film of salt solution, which is small compared with the volume of the nerve, so the composition of the film of salt solution would change rapidly as ions enter or leave the nerve. This situation is quite unlike that obtaining in the animal, where nerves are surrounded by a large volume of freely-circulating fluid. Therefore, measurement of conduction velocities with the nerve in a large volume of bathing solution would be expected to give a more meaningful indication of conditions within the animal, than would measurements made in oil. Another reason for not using oil was that a layer of oil on top of the physiological solution was found to be disadvantageous when the solution had to be changed during the course of an experiment. To prevent the nerve drying out between readings, it was completely immersed by upwards displacement of the bathing solution. The volume of the bathing solution was 100 ml. in all experiments.

A diagrammatic representation of the circuit used for stimulating and recording is shown in Figure 33. A rectangular pulse generator (Grass Model S4 square wave stimulator) with variable duration, frequency and voltage was used as a stimulator. The stimulus was isolated from ground with a Grass Model SIU4 stimulus isolation unit. (The secondary coil of the radio frequency oscillator transformer of this unit has low capacitance

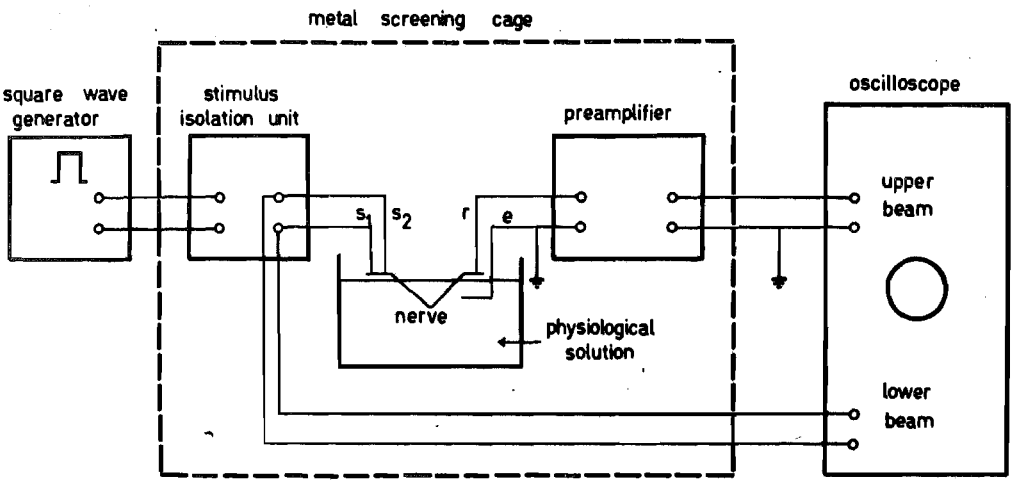


Figure 33: Diagrammatic representation of the circuit used for stimulating and recording.

and conductance to ground; the secondary voltage is rectified and filtered and the stimulus isolation output is then nearly identical in waveform with the modulating pulse, but isolated from ground.)

The signal from the recording electrodes was fed into a Tektronix Type 122 preamplifier, which is an ac-coupled three stage amplifier, the first two stages being operated push-pull. A voltage gain of approximately 1000x was obtained, and this signal was then displayed on a Tektronix Type 502 Dual Beam Oscilloscope with a long-persistence (P 7) phosphor. The stimulating pulse was monitored on the second beam of the C.R.O.

The time taken for the conduction of a spike along a nerve (for calculation of conduction velocity), and the size of the spike, were measured directly by reading the position of the trace on the oscilloscope screen. (The graticule of the oscilloscope was marked at 2mm. intervals. The greatest source of error in reading the position of the trace on the tube was one of parallax, due to the space between the graticule and the cathode-ray tube.) To enable easy visual comparison of the compound action potentials of different nerves, the signals were traced on to cellophane and then transferred to graph paper (metric, in 2mm).

4.2.2 Experiments

The cerebropedal and cerebropleural connectives were dissected out from Scutus specimens which had not been anesthetized. When not under any tension, these connectives are about 2 cm. long, but they can be stretched to almost twice that length and still retain their power to conduct. Nerve cell bodies are present only in the regions of the connectives adjacent to the ganglia (Section 3, Fig. 27); these ends were gripped in the

forceps during the electrophysiological experiments, so that nerve cell bodies were not present in the length of the nerve along which the nerve impulses were passed. The connective, in the region between the electrodes, consisted of a bundle of nerve fibres and scattered neuroglial cells, surrounded by a thin sheath and, at the gain used in these experiments, no spontaneous impulses were detected.

After removal from an animal, nerves were immediately placed in a 'normal' physiological solution*. If a nerve was not to be used for an experiment immediately, it was placed (in about 20 ml. of physiological solution) in a temperature-controlled room at 4°C. Nerves kept at this temperature retained their properties of excitability for at least five hours. They were removed from the cold room at least 30 minutes before the commencement of an experiment, to ensure that the nerve was completely equilibrated to the temperature of the room in which the experiment was to be carried out. When this was not done, slow warming of the nerve during the course of the experiment affected the results considerably.

After being fixed in position in the bath containing normal physiological solution, nerves were left for at least five minutes before measurements were recorded. (No directional difference was observed in the propagation of the nerve impulses; in some experiments the stimulating electrodes were placed on the end of the connective adjacent to the cerebral ganglion while, in other preparations the recording electrodes were adjacent to the cerebral ganglion so that impulses were induced to pass along the connective in the opposite direction.)

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* The 'normal' physiological solution had an ionic composition based on the concentrations of ions in the blood, determined in Section 2 - see Appendix IV.

Measurements were then made at five-minute intervals, until consecutive readings showed a constancy in the form of the action potential. The mean of the final three of these readings was taken as the "base line" for that particular experiment.

(a) Examination of the electrophysiological properties of the connectives

Before control experiments (b-d) were carried out, a series of preliminary experiments was made to study the form of the action potential (A.P.) in the two connectives, the conduction velocities* of the various

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* Conduction velocity. Although the most accurate way of measuring the conduction velocity is to measure the distance and conduction time between two sets of recording electrodes, this method was not used in this study, as the intention was to study the effect of the bathing medium on the conduction in the nerve. This required that the portion of the nerve along which the conduction velocity was to be measured should be surrounded by the physiological solution. Therefore, it was considered that an estimate of the conduction velocity by measurement of the time elapsed between the appearance of the stimulus artifact and the action potential would give a satisfactory indication of the effects of change in the composition of the bathing medium on conduction in the nerve. This time includes time taken up in initiating the impulse and therefore it does not enable calculation of the absolute conduction velocity. (The time taken to initiate the impulse may vary also, but it is quantitatively insignificant compared with the time for the impulse to travel along the nerve, and it may be disregarded.) For ease of reading, in this work the length of the nerve between the stimulating electrode and the recording electrode, divided by the time elapsing between the appearance of the stimulus artifact and that of the action potential, is referred to as conduction velocity.

components of the A.P., the effects of variable duration and voltage upon this compound action potential, etc.

(b) Effect on the conduction velocities and on the heights of the various components of the action potential of time elapsing from the initial recording

A nerve was fixed in position and kept at room temperature (about 21°C), and measurements of the conduction velocities and heights of the different components of the action potential were made at intervals over a three hour period. The stimulating pulse was constant in duration and voltage.

(c) Effect of nerve stretch on conduction velocity

A nerve was fixed in position, so that it was slack between the electrodes, and the conduction velocities of the various components of the action potential were measured. The nerve was then gradually stretched and the conduction velocities measured at varying degrees of extension of the connective, until a point of critical tension was reached and conduction failure occurred. Care was taken to keep the electrodes in the same position on the nerve, for some investigators have found that conduction velocity can alter in different parts of a nerve, possibly due to tapering of the fibres, or persistent folding in the proximal region of the nerve, while the fibres in the middle region become more fully stretched (Nisbet, 1961).

(d) Effect of temperature on conduction velocity

Nerves and the physiological bathing solution were taken straight from a cold room at 4°C, and measurements of conduction velocities were made as the solution gradually warmed to room temperature (19-20°C).

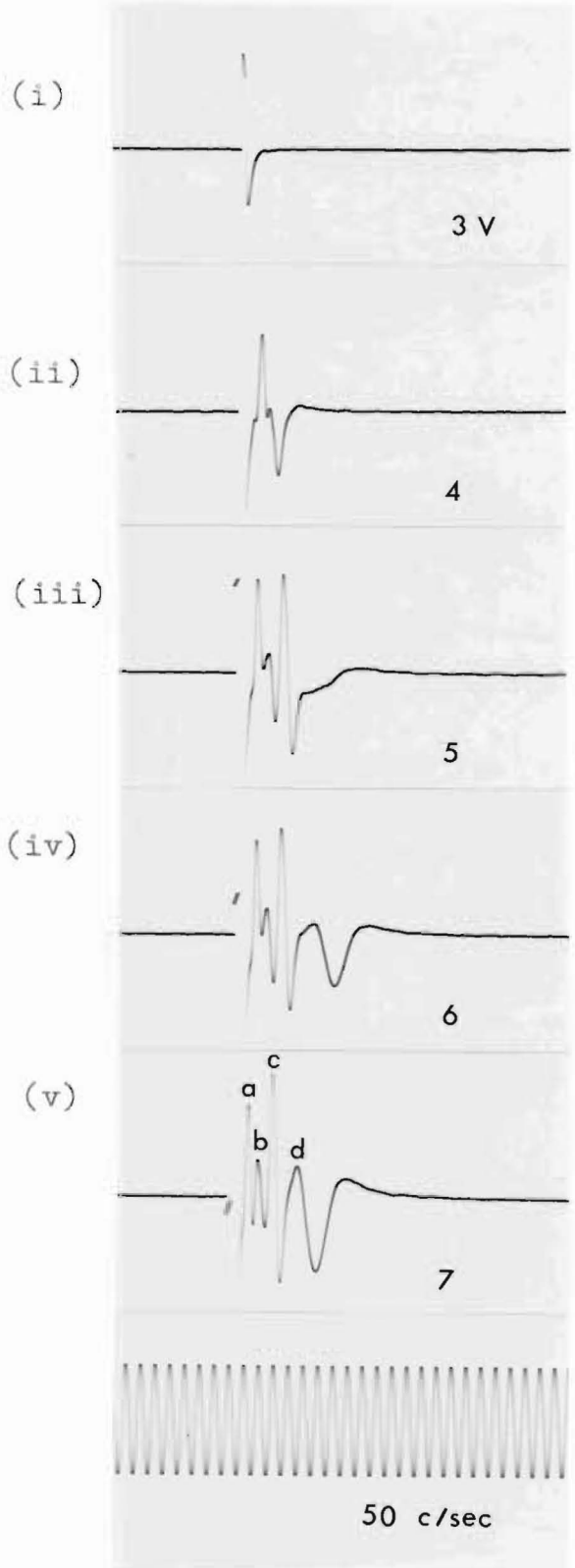

Figure 34 (i) - (v)

Photographs of the action potential of a cerebropleural connective following stimuli of increasing voltages at constant duration (1 msec).

(i) Stimulus artifact alone is visible.

(ii) Stimulus artifact too faint to be recorded. Spikes a and b visible.

(iii) Spikes a, b and c present.

(iv) and (v) Spikes a, b, c and d all visible.

Photographs were taken with a Grass Model C-4 Oscilloscope Recording Camera using Linagraph paper No. 44 "Kind 1505", and a film speed of 100 mm/sec.

Temperatures of the solution were taken adjacent to the nerve. The solution was mixed and aerated with a fine stream of air and, under these conditions, warming of the bathing solution to room temperature took up to about one hour.

(e) Effect of dilution of the medium on conduction velocity

A connective was placed in normal physiological solution to obtain the "base-line" conduction velocity; the normal physiological solution was then replaced with an equal volume of a dilute solution (either Solution 2 ("75%"), or Solution 3 ("environmental dilution")), and readings were made over a 30 minute period. Finally, the normal solution was returned to the bath and the conduction velocity of the nerve was measured again. As a control, to determine the physical effect of changing the medium, experiments were conducted with exactly the same procedure as in the dilution experiments except that, instead of replacing the normal physiological solution with one of different concentration, the same solution was returned to the bath.

4.3 RESULTS

(a) Electrophysiological properties of the connectives

(i) With adequate parameters of stimulation, the action potential of both the cerebropedal and cerebropleural connectives consists of usually four, but in some cases five or six, components (Fig. 34). The velocities of four of these components were similar for both connectives, but the cerebropleural connective had some faster-conducting fibres. A small action potential, which often disappeared as the preparation aged, revealed

the presence of the slowest conducting fibres. To enable comparison and grouping of results from different preparations, and for ease of reference, these characteristic components of the compound action potential were classified as spikes a, b, c, d, and e. Each of these spikes corresponds to the action potentials of a group of fibres with similar conduction velocities. Spike a represents the action potential of the fastest conducting fibres present in the cerebropleural connective, and spikes b-e represent the progressively more slowly conducting fibres, present in both connectives. Conduction velocities of spikes a-e of five cerebropleural and of b-e of five cerebropedal connectives were compared and the results are shown in Table 6.

TABLE 6

Mean values for the conduction velocities of the spikes of the compound action potentials of five cerebropedal and five cerebropleural unstretched connectives. (Medium = normal physiological solution. Mean temperature = 21.6°C)

	Conduction velocity. (m/sec)				
spikes / connective	a	b	c	d	e
c-pedal conn.		0.384	0.264	0.193	0.127
c-pleural conn.	0.601	0.389	0.271	0.193	0.133*

* Shown in only two preparations.

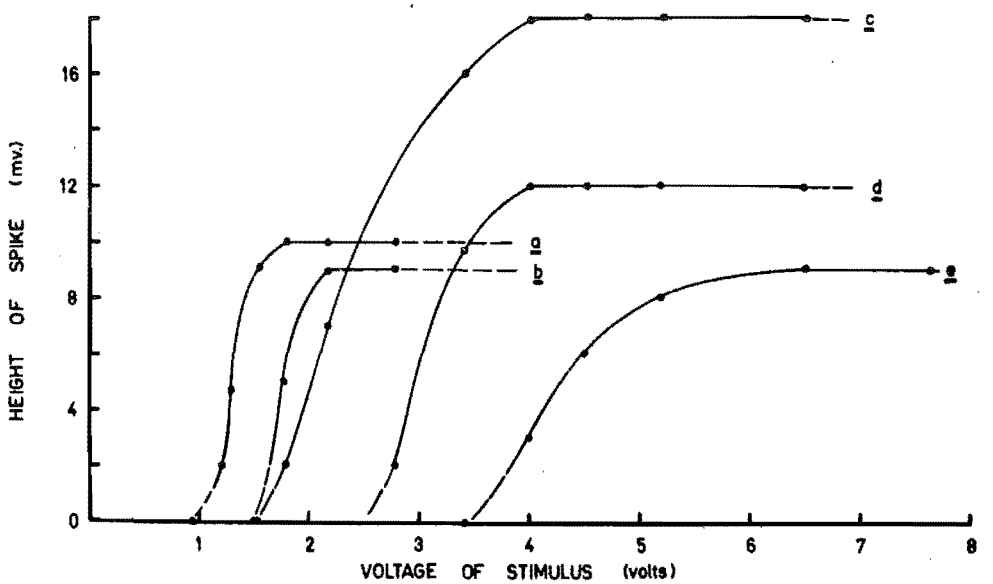

Figure 35: Heights of spikes a - e of a cerebropleural connective as the voltage of the stimulus is increased and the duration (1 msec) and the frequency (1 c/sec) kept constant. (The preamplifier gain was set at 100x, and the Y-axis shows the height of the spikes as read on the oscilloscope screen.)

Because of the very similar nature of the conduction properties of the two connectives, in later experiments results from both cerebropedal and cerebropleural nerves were combined. The mean values, with standard deviations, for each of the main spikes of the action potentials of the unstretched connectives in normal physiological solution, at room temperature, are given in Table 7.

TABLE 7

Conduction velocities of spikes a-e of the action potentials of the cerebropedal and cerebropleural connectives. (Temperature range 17.2-22.7°C)

Spike	Conduction velocity (m/sec)		N
	Mean	S.D.	
a	0.635	0.034	8
b	0.379	0.026	20
c	0.266	0.014	19
d	0.195	0.014	18
e	0.128	0.009	10

The maximum velocity which was recorded from any preparation was 1.1 m/sec, a value obtained for an a spike of a stretched cerebropleural connective in normal physiological solution at 23.2°C.

(ii) The order of spikes according to velocity of conduction usually differed from the order according to size, though relative heights of the different components of the action potential varied from preparation

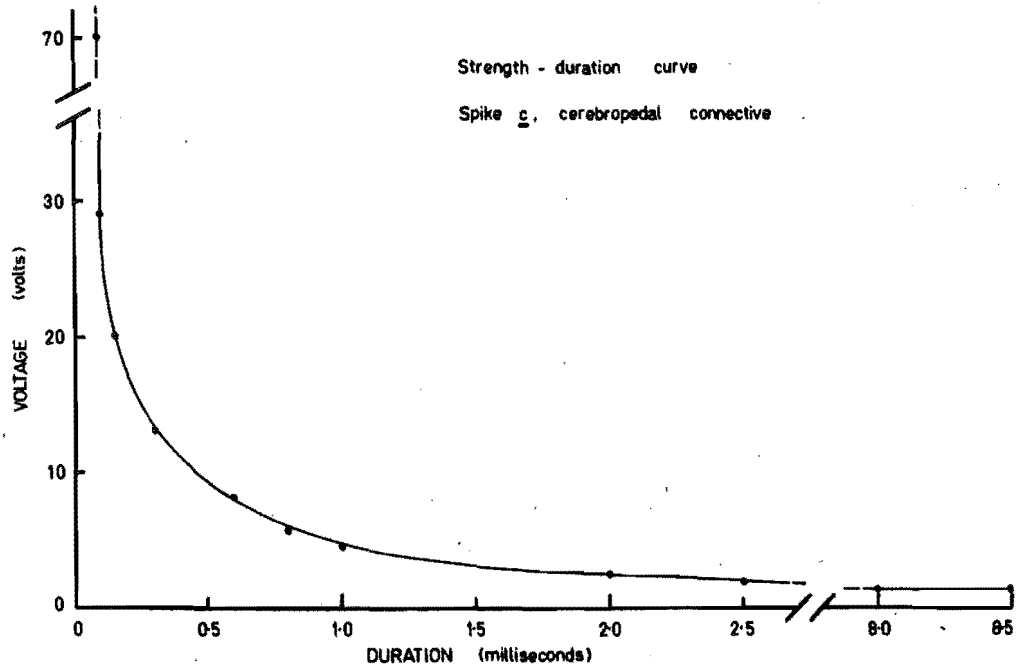

Figure 36: Strength-duration curve of a c spike of a cerebropedal connective. The conduction velocity of the spike was 0.27 m/sec.

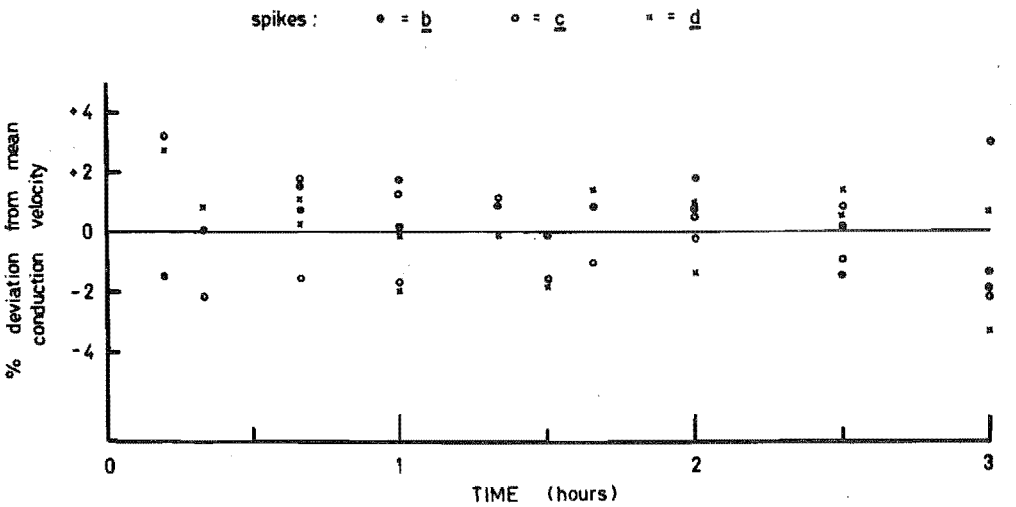

Figure 37: Effect on the conduction velocities of the spikes of two cerebropedal connectives of time elapsing from fixing the nerve in position on the electrodes. (The changes in the heights of the spikes of these connectives are shown in Figure 38).

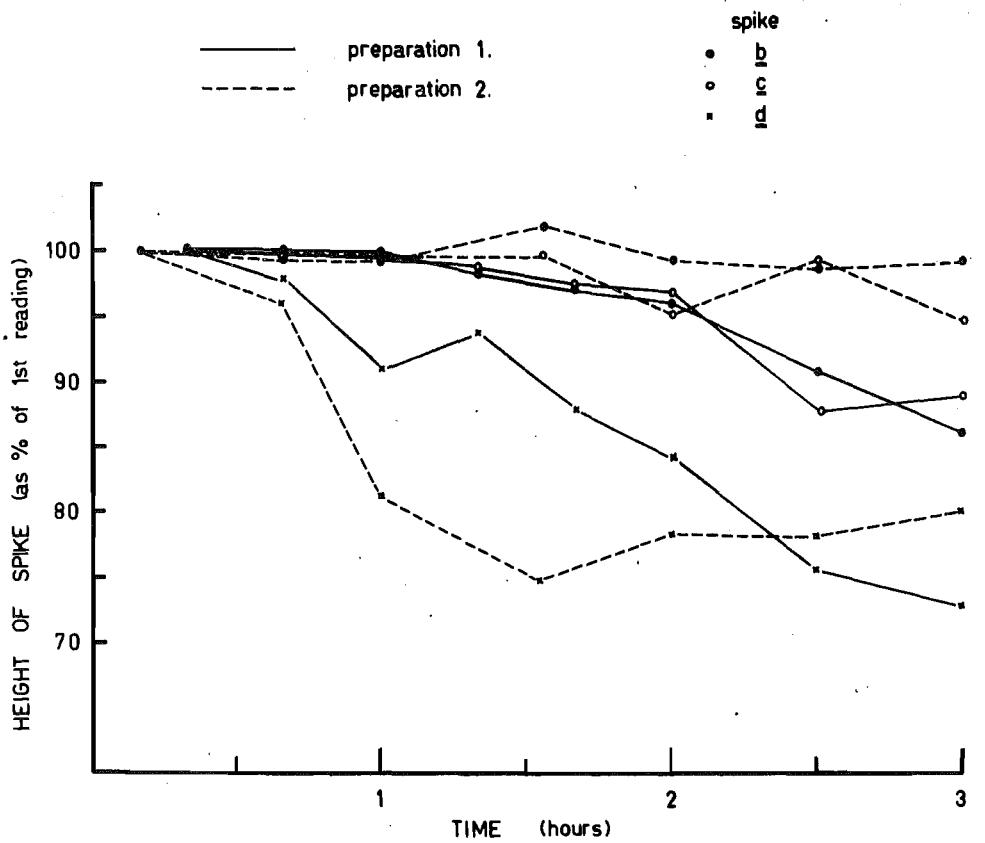

Figure 38: Effect on the height of the spikes of two cerebropedal connectives of time elapsing from fixing the nerve in position on the electrodes. (The changes in the conduction velocities of the spikes of these connectives are shown in Figure 37.)

to preparation (Fig. 34).

(iii) The rheobase differed for fibres of different conduction velocities, and the proportions of the individual components a-e of the overall action potential changed with increase of the stimulus voltage at constant pulse duration (Fig. 35). From this figure it is also clear that at low voltages the fastest component, spike a, was largest, but as the voltage was increased the sizes of the slower components increased until the maximum values of spikes c and d were greater than that of spike a.

(iv) A strength-duration curve for a c spike of a cerebropedal connective nerve preparation is shown in Figure 36.

(b) Effect on the conduction velocities and on the heights of the various components of the action potential of time elapsing from the initial recording

During the first ten minutes after a nerve had been fixed in position, the form of the action potential sometimes showed considerable variations, but after this initial 'settling down' period the conduction velocities of the different fibres remained fairly constant for at least two hours (Fig. 37). However, the heights of the spikes often showed a decrease with time (Fig. 38) when the stimulus was kept constant. Greatest decreases were found for the slowest conducting fibres (spikes d and e), probably because the voltage of the stimulus was closer to the rheobase for these fibres than for the faster fibres, and a larger stimulus would have been required to cause firing of the spike as the nerve became fatigued. From Figures 37 and 38 it can be seen that, after about two hours at room temperature, the

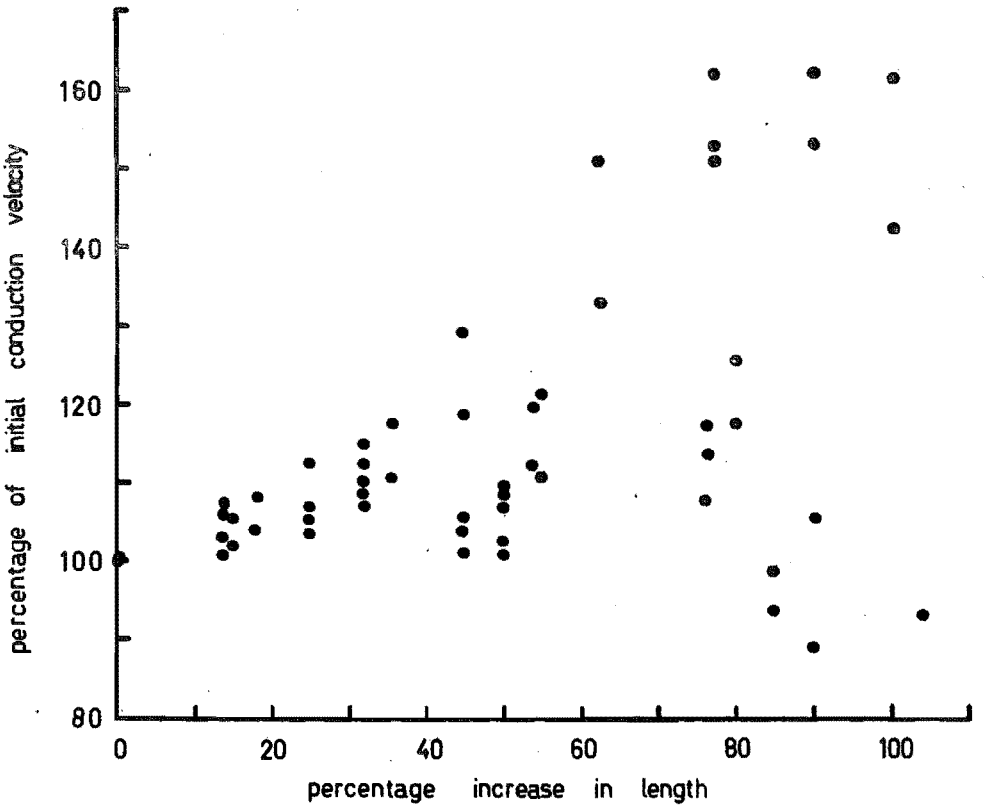

Figure 39: The effect of nerve stretch on conduction velocity. Results for 20 spikes (six preparations) are shown.

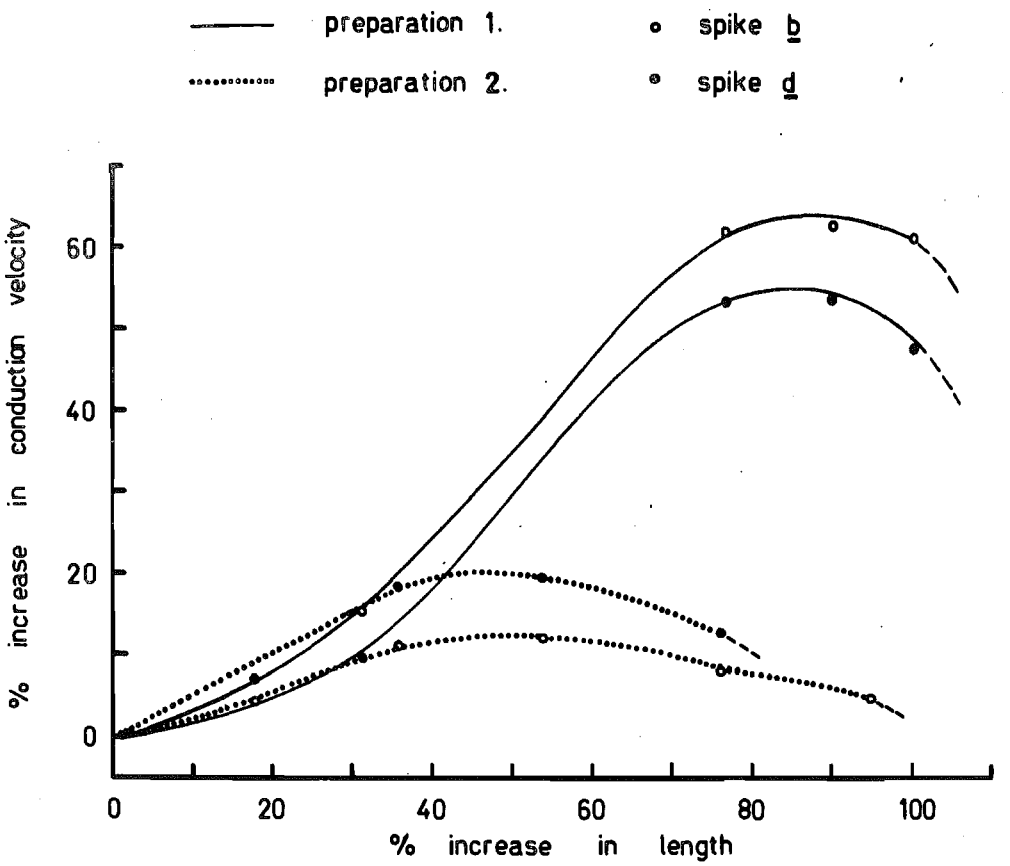

Figure 40: Effect of nerve stretch on the conduction velocity of spikes b and d in two cerebropedal connectives.

conduction properties of the nerve often began to show greater deviations from the initial values but, as dilution experiments were confined to about one hour for each preparation, this variability shown after two hours was not relevant. As the conduction velocity remained much more stable over a period of time in the control experiments than did the height of the action potential, the former was chosen as the parameter to measure during the dilution experiments. The different spikes showed independent variation and so were measured individually in all experiments.

(c) Effect of nerve stretch on conduction velocity

Figure 39 shows the relationship between the apparent conduction velocity and the length of the nerve, as a nerve is gradually stretched. Results for 20 spikes are shown, and in each case the conduction velocity has been calculated as a percentage of the initial velocity in the unstretched nerve. Curves for the fastest and slowest spikes from each of two preparations are shown in Figure 40. As nerves were stretched an apparent increase in conduction velocity was shown by all groups of fibres. A maximum conduction velocity was reached, and this could be as great as 160% of the initial value in the unstretched nerve. The percentage increase in length of the nerve at which this maximum occurred, and the degree of increase in conduction velocity, varied in different preparations; this would be expected, because the state of tension taken as "zero" stretch in different preparations would not be exactly the same. When a nerve was stretched to about twice its original length there was invariably conduction failure, usually preceded by a reduced rate of conduction.

The results of these experiments show the importance

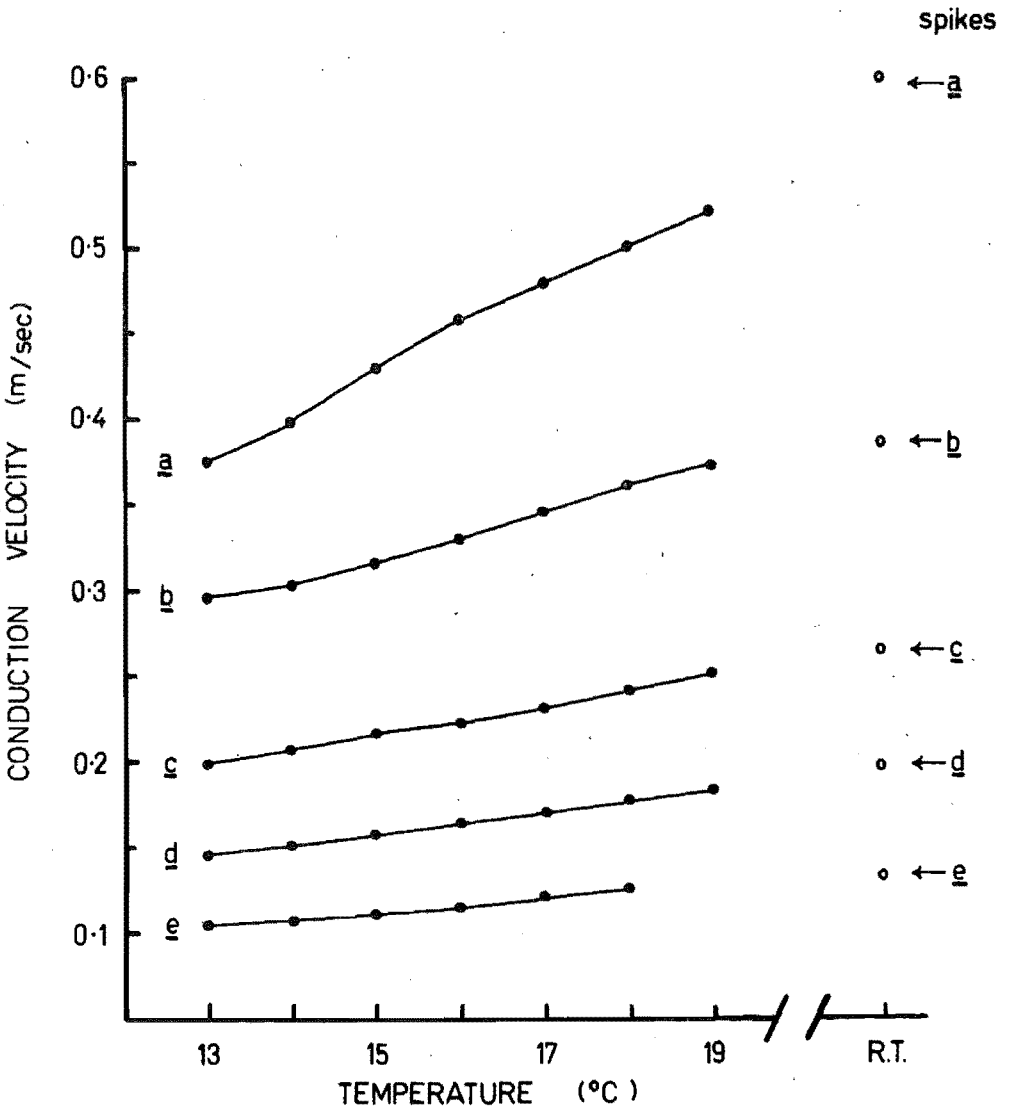

Figure 41: Effect of temperature rise (13-19°C) of the surrounding medium on conduction velocity. For spikes b - e, N = 5, for spike a, N = 1. Also shown on the graph (at right) are mean conduction velocities of the spikes (N = 20) measured at room temperature (R.T. range 17.2 - 22.7°C).

of keeping constant the tension, and therefore position, of a nerve throughout the course of an experiment. They also show the difficulty in getting meaningful results by direct comparison of the absolute conduction velocities of different preparations.

(d) Effect of temperature on conduction velocity

Figure 41 shows the increase in conduction velocity of all spikes of the connective, caused by an increase in temperature from 13° to 19°C. Also included in the figure are the mean values of the conduction velocities of the spikes of the compound action potential, obtained from 20 preparations, measured at room temperature (17.2-22.7°C).

The temperature coefficients of the five spikes, expressed as Q_{10} values, are given in Table 8. When measurements were being made, a small portion of the nerve, in the vicinity of the electrodes, was not surrounded by the physiological solution, so that the temperature along the whole nerve would not be completely uniform. However, most of the nerve was bathed in the fluid and the Q_{10} values calculated here should give an indication of the extent to which temperature affects conduction.

TABLE 8

Q_{10} values for the conduction velocities for the five major components (spikes a-e) of the action potentials of six c-p connectives, where

$$Q_{10} = \frac{K_1}{K_2}^{\left(\frac{10}{T_1 - T_2}\right)}$$

In this experiment T_1 = 13°C,
T_1 = 19°C.

Spike	Mean conduction velocity (unstretched nerve) at 19°C (m/sec)	Q_{10}	
		Mean	Range
a	0.520	1.69*	
b	0.370	1.48	1.36-1.63
c	0.252	1.50	1.38-1.68
d	0.186	1.47	1.33-1.63
e	0.126 (18°C)	1.55	1.44-1.75

* Single value (i.e. N=1)

(e) Effect of dilution of the medium on conduction velocity

Table 9 shows the variation in conduction velocity found when a nerve preparation was subjected to the experimental procedure used in the dilution experiments, but where the same physiological solution was returned to the bath after each 'solution change' procedure (i.e. it shows the results of the control experiments to check effects of mechanical disturbance due to changing the bathing solution). The data show that,

once the nerve preparation had stabilised after a short period in the bath, subsequent changes of the fluid did not alter the mean conduction velocity, although the velocities did show more variability as the experiment continued.

TABLE 9

Variations in the conduction velocities of spikes when the bathing medium is changed twice during a one hour period. (These values are used for the control curves in Figures 42 and 43.)

Reading No.	Time (min.)	Conduction velocity		
		% of mean of 1st 3 readings	S.D.	N
1	0	101.0	1.43	7
2	5	99.4	1.23	8
3	10	99.5	1.40	8
s o l u t i o n c h a n g e				
4	12	99.6	1.00	8
5	15	100.3	1.45	8
6	20	99.6	2.21	8
7	25	99.0	2.11	8
8	40	99.5	3.80	8
s o l u t i o n c h a n g e				
9	42	99.4	3.72	8
10	50	99.5	3.82	8

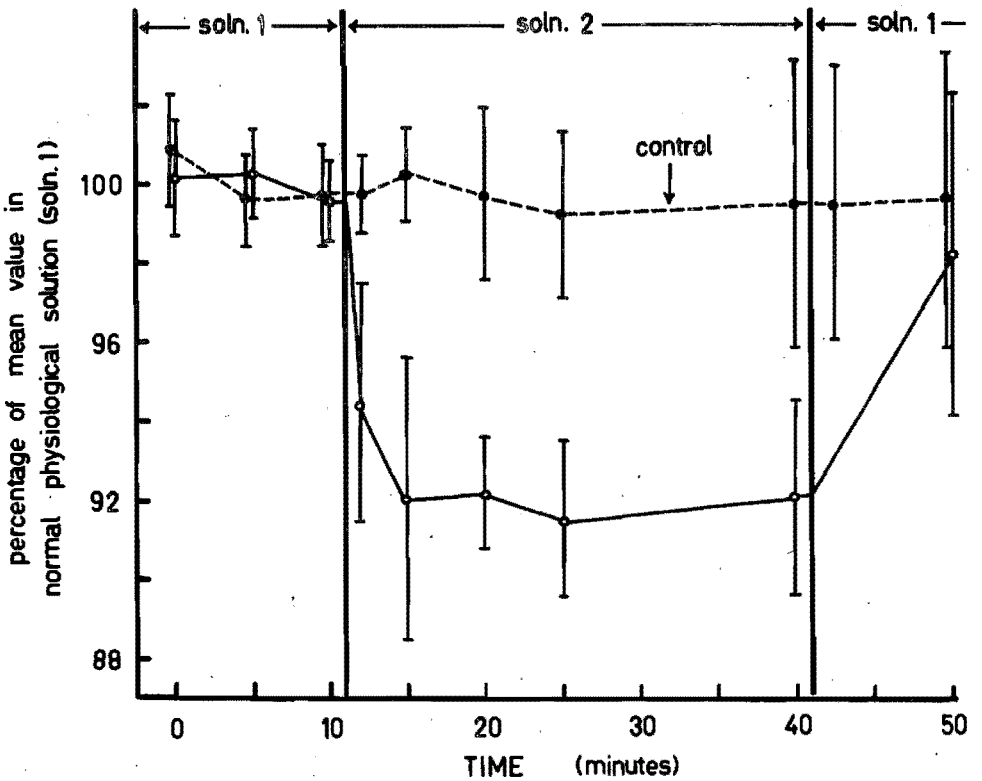

Figure 42: Effect of dilution of the medium on conduction velocity. (Solution 2 has cation composition similar to that of the blood of animals kept in 75% sea water for 24 hours.) The control curve is the mean curve for 8 spikes, and the curve for nerves in the dilute solution is the mean for 24 spikes. Standard deviation ranges are shown for both curves.

(i) Dilution to Solution 2 ("75%"). Nerves placed in this solution with cation composition similar to that of the blood of animals kept in 75% sea water for 24 hours, showed a slight decrease in conduction velocity. For each spike, values were calculated as a percentage of the initial conduction velocity in normal physiological solution. Spikes a-e all showed similar decreases, so values for all spikes were averaged together. A curve of the mean values (with standard deviation ranges) of the conduction velocities of spikes from six connectives is shown in Figure 42. The decrease below the control was statistically highly significant ($P < 0.005$) for each of the times at which measurements were made when the nerve was in dilute solution. The conduction velocity increased again when the normal physiological solution was returned to the bath, but the initial velocity was not always regained.

The larger standard deviations of measurements made immediately after the solutions were changed reflect the variation in time taken by different nerves to show an alteration in conduction velocity when subjected to a change in the concentration of the surrounding medium. This variation in time of physiological response is probably partly due to differences in the permeability of the sheath surrounding the connective. The thickness of this sheath is not constant from animal to animal.

(ii) Dilution to Solution 3 ("environmental dilution"). Experiments in which the normal physiological solution was replaced with one (Solution 3), with cation concentration equal to the minimum value of the blood reached in animals subjected to changing

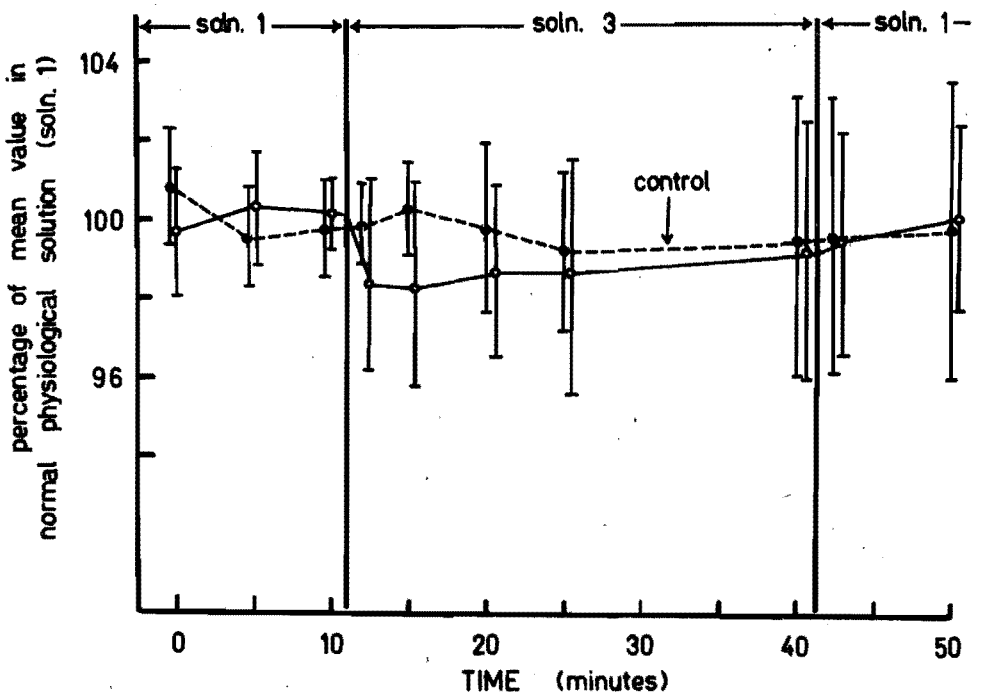

Figure 43: Effect of dilution of the medium on conduction velocity. (Solution 3 has a cation concentration equal to the minimum value of the blood reached in animals subjected to changing salinity simulating conditions in the Heathcote-Avon Estuary.) The control curve is the mean curve for 8 spikes, and the curves for nerves in the dilute solution is the mean for 23 spikes. Standard deviation ranges are shown for both curves.

salinity imitating environmental conditions in the Heathcote-Avon Estuary, gave no conclusive results (Fig. 43). Although the curve for the mean value of the conduction velocities of nerves in the dilute solution is slightly below that of the controls, the differences between the data points are not significant at the 5% level, except for the measurements taken after a connective had been in the dilute solution for four minutes, when $P < 0.025$. At no point is the mean conduction velocity of the nerve in dilute solution less than 98% of the initial value in normal physiological solution, and this is within the range of experimental error.

4.4 DISCUSSION

The aspects of the spike which are needed to obtain a picture of the activity of a nerve are

(1) the form of the spike (amplitude and time course),
(2) the recovery cycle (refractory periods),
(3) threshold voltage as a function of duration of stimulus (in the case of abruptly applied shocks - rectangular pulses) and of slope (in the case of slowly rising stimuli), and
(4) the conduction velocity.

The last parameter is the only one which was measured in all experiments in this work, and is the one which will be considered in the following discussion.

Maximum conduction velocity values reported for the nerves of gastropods show a considerable range, and those obtained for Scutus fall within this range. The lowest maximum values found were 0.35-0.44 m/sec for the pedal nerves of Ariolimax (Carlson, 1911). The highest value found was 3 m/sec for fibres in the giant

African snail, Archachatina (Nisbet, 1957). This latter value was associated with a maximum fibre diameter of 40 μ. For Scutus the maximum values were 1.1 m/sec for stretched, and 0.702 m/sec for unstretched cerebropleural connectives.

(a) Effect of stretch

The effect of the stretch of the nerve on the conduction velocity appears to differ in various species. Animals with relatively fixed dimensions, like vertebrates and arthropods, typically tolerate only a few per cent increase in length, while in other animals, such as gastropods and annelids which normally extend and contract greatly, the nerves can be stretched by a considerable amount and still continue to conduct. In these animals some of the changes in length are enabled by a simple unfolding of the sinuously folded nerve; when the sinuosity is removed the nerve can be still further stretched and still conduct.

Bullock (1945) reported that conduction velocity in the giant fibres of Lumbricus increased as the animal was stretched, but not proportionately, as the elapsed time also increased, while Adey (1951) found that the maximum velocity of the median giant fibre was approximately linearly related to the worm length in the range 15-40 cm. Bullock, Cohen and Faulstick (1950) found that stretch caused an increase in conduction velocity in the giant fibres in about 12% of the cases. They observed that, when unstretched, the fibre was folded within the ventral cord. As the animal was stretched the fibre unfolded without any changes in fibre diameter and the velocity appeared to increase. However, when the fibre had been pulled out straight, further stretching reduced the fibre diameter up to one half of its

original value, but had no effect on conduction velocity.

For Aplysia, Goldman (1961) found that, as connectives were stretched, the conduction velocity increased to a maximum of up to about 150% of the initial value in the unstretched nerve. The velocity then did not differ appreciably from this maximum value at any length (up to 350%) of the connective greater than that at which the maximum was first reached. However, in the slug Ariolimax, stretching the pedal nerves brought about no significant change in conduction velocity of the fastest fibres (Jenkins and Carlson, 1904; Turner, 1951), although in some cases it caused a rather marked change in the configuration of the action potential.

In contrast to the effects on other species, cited above, the usual effect of stretch on the connectives of Scutus was an initial apparent increase in the conduction velocity to a maximum which, in some cases, was maintained while the nerve was stretched still further; this was then followed by a decrease in velocity before final conduction failure. No histological studies were made of nerves subjected to various degrees of tension, but it would seem, à priori, that the initial apparent increase in conduction velocity could be explained by the unfolding of microfolds in the walls of the fibres as the nerve was stretched. This unfolding would bring an increase in the measured length of the nerve, while the actual surface areas of the membranes of the fibres along which the impulses passed, and the 'real' diameters of the fibres, would remain approximately constant. Goldman obtained histological evidence of this phenomenon in Aplysia. The subsequent decrease in conduction

velocity could be explained by the decrease in fibre diameter brought about by extreme stretching although, as was mentioned earlier, Bullock, Cohen and Faulstick found that, for Lumbricus, this was not the case. Overstretching might affect the actual excitability and responsiveness of the membrane by affecting one of the passive membrane properties such as specific capacitance (e.g. see Martin, 1954).

(b) Effect of temperature

The Q_{10} values for conduction velocities obtained for Scutus in this work are of the same order as those obtained by other investigators for different species. For example, Turner (1953) obtained Q_{10} values of 1.37 (range 1.06-1.83) for the preganglionic fibres, and 1.42 (range 1.00-1.95) for the postganglionic fibres of the pedal nerve of Ariolimax columbianus; the Q_{10} for conduction velocity was similar to those for ganglionic delay and utilization time. Similar Q_{10} values were obtained for conduction velocity in giant fibres of Lumbricus; in the medial fibres Q_{10} = 1.52 (range 1.21-2.12) and in the lateral fibres Q_{10} = 1.46 (range1.13-1.82) (Turner, 1955). Wright (1958) found that, for single crustacean motor axons, the conduction velocity was reduced by a factor of three or four with a temperature reduction of 20°C. For single fibres from a toad, Tasaki and Fujita (1948) reported a Q_{10} value of 1.8 and, using the sciatic nerve of a frog, Gasser (1931) obtained a mean Q_{10} value for conduction velocity of 1.85 (range 1.4-3.0), when measurements were made within the temperature range 10 - 30°C. However, for one preparation the conduction velocity was measured at 5° and 10°C, and the Q_{10} calculated from these measurements was found to be 4.1. The temperature range (5-10°C), in which this experiment

was carried out, was much lower than that (25-30°C) at which the frogs had been living, and is below the temperatures which were used in the present work. At these low temperatures, Gasser noted that the action potential declined rapidly and, in some cases, around 5°C there was failure to respond altogether.

As Turner (1955) pointed out, "Because of the probably complex nature of changes in nerves brought about by changes in temperature, the significance of the Q_{10} for the conduction velocity is hard to evaluate. At present, the roles played by such factors as ionic diffusion, active ion transport, and underlying metabolic effects cannot be adequately assessed."

(c) Effect of dilution of the medium

As Katz (1947) found for the non-medullated nerve fibres of Carcinus, when the electrolyte concentration of the bathing medium is decreased the conduction velocity of a nerve is decreased, but this effect is not very marked when the nerve is surrounded by a large volume of electrolyte solution. At low salt concentrations Katz considered that this reduction could not be accounted for by an increase in external resistivity, and he thought that it was probably due to a change in the membrane properties. However, he found that these changes were only pronounced when there was a salt reduction to less than one third of the normal concentration, unless the fibre was immersed in oil, thereby being surrounded by only a thin film of electrolyte solution.

In Scutus no significant decrease in conduction velocity was measurable when the nerve was exposed to dilutions such as it would be likely to encounter in its

natural environment. However, in conditions which would be found in an animal kept in 75% sea water, the conduction velocity is measurably decreased. The 'sluggishness' and loss of muscle tone of animals kept in 75% sea water could be correlated with the decrease in the powers of conduction of the nerves, which has been found at these concentrations in this work.

In experiments done in this study, the concentrations of all the cations were reduced simultaneously, so it is not possible to conclude whether any one cation produced any particular effect. Investigations into the effects of individual cations have been made by many workers, particularly using squid giant axons and crustacean fibre preparations. As far as conduction of the nervous impulse is concerned, Wright and Tomita (1965) summarized that sodium controlled the spike, potassium controlled the after potential and calcium (and, to a certain extent also, magnesium) controlled the membrane permeability.

4.5 CONCLUSIONS

Scutus is normally a marine animal and is only found in areas of the Heathcote-Avon Estuary which are not subjected to very marked or prolonged dilutions of sea water. Results obtained in this work show that when animals are exposed, in the laboratory, to conditions simulating those which they do experience in their natural estuarine environment, the blood is slightly diluted, but this dilution is not great enough to cause any noticeable difference in the conduction velocity of the nerve fibres. However, when animals are subjected for extended periods to dilutions of sea water (such as 75%) which are more severe than

those experienced in the environment, but are well within the range found along the shoreline in parts of the estuary, the blood is markedly diluted, approaching an equilibrium concentration with the surrounding medium. A nerve placed in a solution with similar electrolyte composition shows a definite decrease in conduction velocity, and it is likely that other parameters of the spike, and excitability of the tissues, such as synaptic transmission and muscular contraction, would similarly be affected. The shell of Scutus is very reduced and therefore it does not afford the physical protection from exposure to a dilute medium as is given by the extensive shells of animals such as limpets; low salinity is probably a major factor in preventing the migration of Scutus into those areas of the estuary which, though they have a similar substrate to the habitats at the culverts under the causeway at McCormacks Bay, are closer to the main river channels and are therefore subjected to lower salinities.

SUMMARY

1. The distribution and habitat of Scutus are discussed. The estuarine habitat was studied more extensively than the marine habitat where Scutus is most commonly found and, in particular, the salinity and temperature changes to which Scutus is subjected in the Heathcote-Avon Estuary were investigated.

2. The sodium, potassium, calcium, magnesium, sulphate, phosphate and protein concentrations of the blood of an animal equilibrated in normal sea water were determined, and the total osmotic pressure of the blood was estimated by determination of the depression of the freezing point. The freezing point and the sodium ion concentration of the blood were found to be very close to values obtained for the sea water in which the animals had been kept, while the potassium, calcium and magnesium concentrations were all slightly higher, and the sulphate slightly lower, than in the sea water. Phosphate and protein were present in only low concentration and evidence was obtained that most of the protein was in the form of haemocyanin.

3. Some responses of Scutus to a change in the concentration of the environment were studied. The concentrations used were 75%, 85% and 115% sea water. The animal was also subjected to salinity changes simulating those which occur during a tidal cycle at the Heathcote-Avon Estuary.

4. Experiments investigating weight changes of animals in dilute media showed that there was an initial weight increase upon transfer to the new medium, and then a weight decrease, associated with deterioration in the physical condition of an animal.

In the hypertonic medium animals lost weight and, after a few hours, two animals showed a slight weight increase.

In a solution of three parts sea water : one part isotonic sucrose, a weight decrease was found.

5. Scutus was found to be poikilosmotic over the range of sea water concentrations which were investigated in this study.

In conditions simulating salinity changes in the estuary, the order of rate of change of decrease in the concentration of the four cations measured in the blood was

$$Ca^{2+} > K^{+} > Na^{+} > Mg^{2+}$$

6. No significant differences in rates of change of blood concentration were found between animals kept in diluted media at 7°C and those kept at 17-19°C.

7. The gross anatomy of the central nervous system is described. It was found to be very like that which has been described for other fissurellids.

8. The microanatomy of the central nervous system was studied, particular attention being paid to the inclusions in the nerve cells. Various staining methods, which have been used by other workers to investigate neurosecretory phenomena, were used to see if neurosecretory material could be identified and if the amount of this material varied when animals were placed in media of different salinities. The numbers of positively stained inclusions in the neurons could not be correlated with salinity changes to which an animal had been subjected prior to fixation.

9. Some electrophysiological properties of the cerebro-

pedal and cerebropleural connectives were studied. With adequate stimulation each of these connectives was found to have a compound action potential whose components (spikes a - e) had mean conduction velocities rangingfrom 0.64-0.13 m/sec.

10. When a nerve connective was stretched the observed conduction velocities of the spikes increased; however, when a connective was stretched to as much as approximately twice its original length there was invariably conduction failure.

11. Q_{10} values for conduction velocities measured between 13° and 19°C were found to fall within the range 1.33 to 1.75.

12. When a nerve was placed in a medium with cation composition close to that found for the blood of an animal which had been in 75% sea water for 24 hours, there was a significant decrease in the conduction velocity of all spikes of the compound action potential. However, when a nerve was placed in a solution with cation composition the same as the minimum concentration of the blood reached in an animal subjected to salinity changes simulating those which occur during a tidal cycle at the Heathcote-Avon Estuary, no significant change from the conduction velocity in normal physiological solution could be detected.

13. Scutus is normally a marine animal, but it is also found in an estuarine habitat where the sea water is not markedly diluted. Results of this work showed that dilutions of sea water of the order experienced by the animal in its natural environment were not great enough to cause a detectable decrease in the conduction velocity of the nerves, but exposure to more hypotonic media for

extended periods resulted in a marked dilution of the blood, which in turn affected conduction in the nerves. It is concluded that the indirect effect which a medium of low salinity has on nervous conduction could be one of the factors which restricts Scutus to areas of the estuary where the sea water is only slightly diluted for a short time during each tidal cycle.

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A P P E N D I X I

CLASSIFICATION

The nomenclature accepted by Powell (1957) for the black sea slug, "rori" of the Maori, was Scutus breviculus. Using Morton's (1958) Classification of Mollusca, based upon that of Thiele (1931-35), the hierachy of groups to which this species has been assigned is:

PHYLUM:	Mollusca
CLASS:	Gastropoda
SUBCLASS:	Prosobranchia
ORDER:	Archaeogastropoda
SUPERFAMILY:	Zeugobranchia
FAMILY:	Fissurellidae
GENUS:	Scutus Montfort, 1810
SPECIES:	S. breviculus (Blainville, 1816)

The genus Scutus was first described by de Montfort (1810, p. 58) - "Genre Pavois, en latin, Scutus". (This generic name was based on Patella in Chemnitz (1795, volume XI, p. 181, table 197, figure 1918)). The description given by de Montfort for Scutus antipodes is "Coquille libre, univalve, en bouclier alongé et aplati; sommet indiqué, apparent, placé au tiers du dos, et en arrière; arrondie dans sa partie postérieur, anterieurement tronquée; bord uni." (Although the New Zealand species has been called S. antipodes by some authors, this specific name is now used solely for one of the Australian species.)

The specific name breviculus of the New Zealand species was introduced, under the generic name Parmophorus, by de Blainville (1816, p. 28).

There has been some confusion in the taxonomy of this animal, and over the past 160 years it has been referred to by many different generic names. Adams (1851), in a monograph on the genus Scutus, lists the following alternatives for the generic name.

Parmophorus - Blainville (also Parmaphorus, Parmorphorus)
Dascinus - Rafinesque
Scutellites - Auct.
Scutum - Sowerby jr
Parmophora - Desmarest
Emarginula - Sowerby
Patella - Lamarck

Tobler (1902), in a paper on the anatomy of Parmophorus intermedius, lists Subemarginula and Tugalia as being synonyms for Parmophorus.

The specific name used for the New Zealand species of Scutus has also been variable. For instance, Hutton (1880) called this species Parmophorus unguis while Suter (1913), in his "Manual of New Zealand Mollusca", used Scutus ambiguus. Scutus breviculus has also been called Scutus antipodes, S. unguis, Parmophorus breviculus, P. australis, P. elongatus, P. intermedius, Patella ambigua, Patella unguis, Subemarginula intermedia, S. parmophoroidea, Tugalia elegans, T. cinerea, T. ossea and T. australis, by various authors.

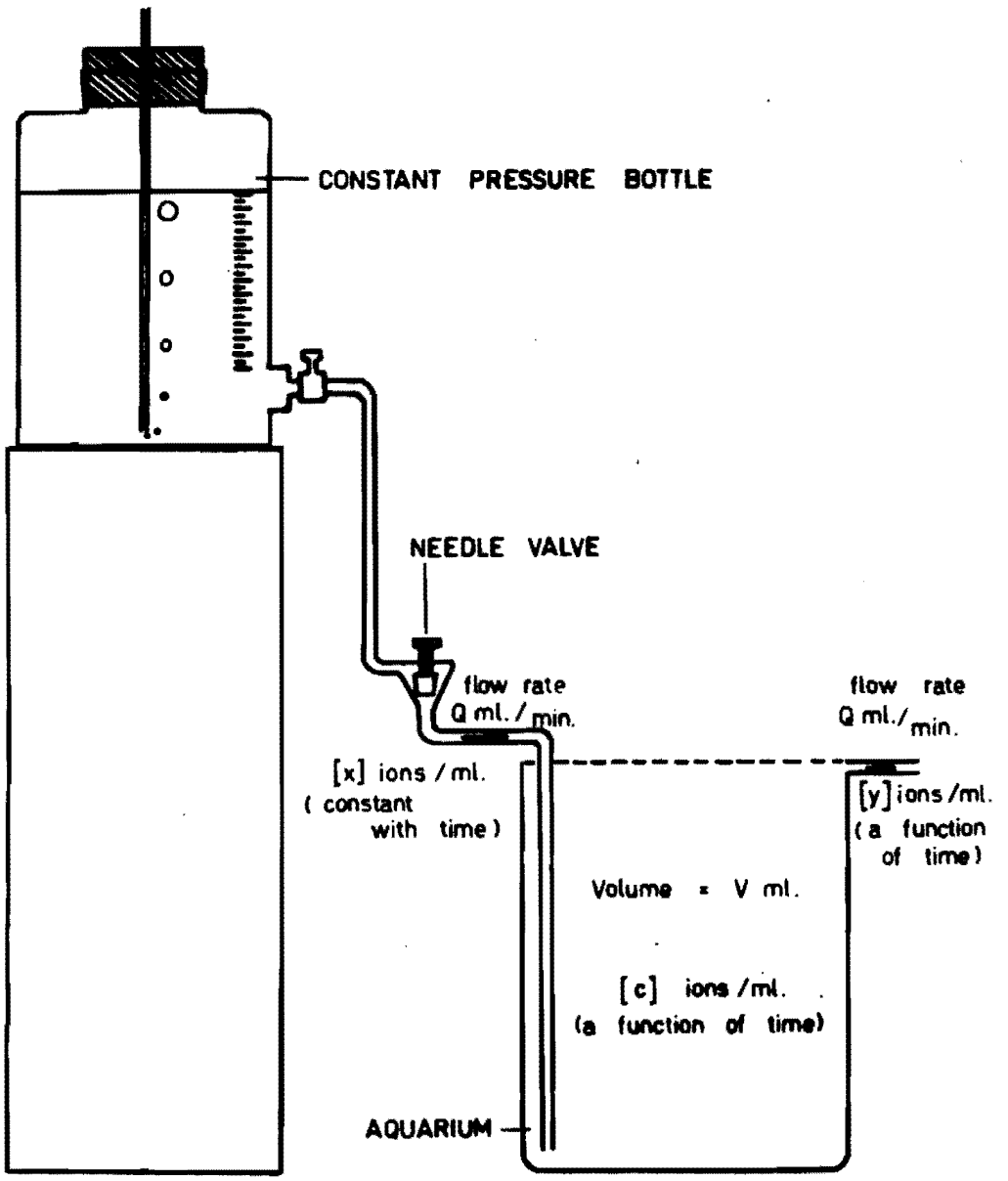

Figure 44: Experimental set-up used to obtain a predictable gradual dilution or concentration of a solution in an aquarium. The solution was continuously aerated and mixed by air being bubbled through a diffusion block.

A P P E N D I X II

CHANGING THE CONCENTRATION OF SEA WATER ALONG A SPECIFIED CONCENTRATION GRADIENT

Figure 44 illustrates the experimental set-up used to obtain a specified gradual dilution, or concentration, of a solution in an aquarium. Wells and Ledingham (1940) published a method in which a rhythmic muscle preparation from a polychaete was exposed to a fluid whose composition "drifted" slowly and steadily along an accurately predictable curve, whose steepness could be varied. The principle of the method described here is the same.

1) Assume that, with constant mixing in the aquarium, the concentration [y] of ions in the outflowing liquid is equal to the concentration [c] of ions in the solution in the aquarium.

i.e. Let $[c] = [y]$

2) The volume (V) of the aquarium was taken as being that volume of fluid which was required to fill the aquarium completely, when it contained two animals of average size. The sizes of the animals used in different experiments varied, but the difference between the sum of the volumes of any two animals used in a particular experiment and the "average" volume allowed for in the initial calculations, was never more than 1.5% of V.

The change in concentration of ions in the aquarium,

$$\frac{dc}{dt} = \frac{Qx - Qc}{V}$$

$$= \frac{Q}{V}(x - c)$$

Integrating, $\int_{C_i}^{C_f} \frac{dc}{(x - c)} = \frac{Q}{V}\int_{T_i}^{T_f} dt$

$$\log_e (x - C_i) - \log_e (x - C_f) = \frac{Q}{V}(T_f - T_i)$$

where C_i = initial concentration of ions in aquarium

C_f = final concentration of ions in aquarium

x = concentration of ions in the fluid being run into the aquarium

T_i = initial time

T_f = final time

Q = flow rate

V = volume of aquarium

Using the above equation flow rate (Q) for dilution of sea water in the aquarium from 100% to 85% in 30 minutes was calculated as follows:

$$C_i = 100$$

$$C_f = 85$$

$$x = 0$$

$$T_f - T_i = 30 \text{ minutes}$$

$$V = 11000 \text{ ml.}$$

Therefore, $$Q = (-\log_e 100 + \log_e 85)\ \frac{11000}{30}$$

$$= (-4.6052 + 4.4427)\ \frac{1100}{3}$$

$$= 0.1625 \cdot \frac{1100}{3}$$

$$= \underline{59.5 \text{ ml./min.}}$$

This flow rate was obtained by adjustment of the screw control on the needle valve (see Figure 44).

Using the same flow rate (59.5 ml./min.) the concentration (x) of the solution in constant pressure bottle, to restore the sea water from 85% to 100%, was calculated as follows:

$$C_i = 85$$

$$C_f = 100$$

$$T_f - T_i = 30 \text{ minutes}$$

$$Q = 59.5 \text{ m./min.}$$

$$V = 11000 \text{ ml.}$$

$$\log_e (x - 85) - \log_e (x - 100) = \frac{59.5}{11000} \times \frac{30}{1}$$

$$\log_e \left[\frac{x - 85}{x - 100}\right] = 0.1623$$

$$\frac{x - 85}{x - 100} = 1.176$$

$$x = \underline{185.3}$$

185% sea water was obtained either by boiling down natural sea water or by making an artificial solution, the composition of which is shown below.

Weights of compounds used to prepare a solution with composition 185% that of sea water collected from Lyttelton (see p. 41).

Cation	Lyttelton sea water mM/l.	185% mM/l.
sodium	487.00	901
potassium	10.87	20.11
calcium	11.56	21.14
magnesium	47.99	88.78

Compound	g/l.
NaCl	46.2796
KCl	1.4985
$CaCl_2.6H_2O$	4.681
$MgCl_2.6H_2O$	18.038
$Na_2SO_4.10H_2O$	16.589
$NaHCO_3$	0.389
$NaBr.2H_2O$	0.146

Weights of $NaHCO_3$ and $NaBr.2H_2O$ are calculated from those suggested by Welsh and Smith (1960 p. 159) for making up artificial sea water. Quantities of other chemicals are calculated from figures obtained from analysis of sea water collected at Lyttelton.

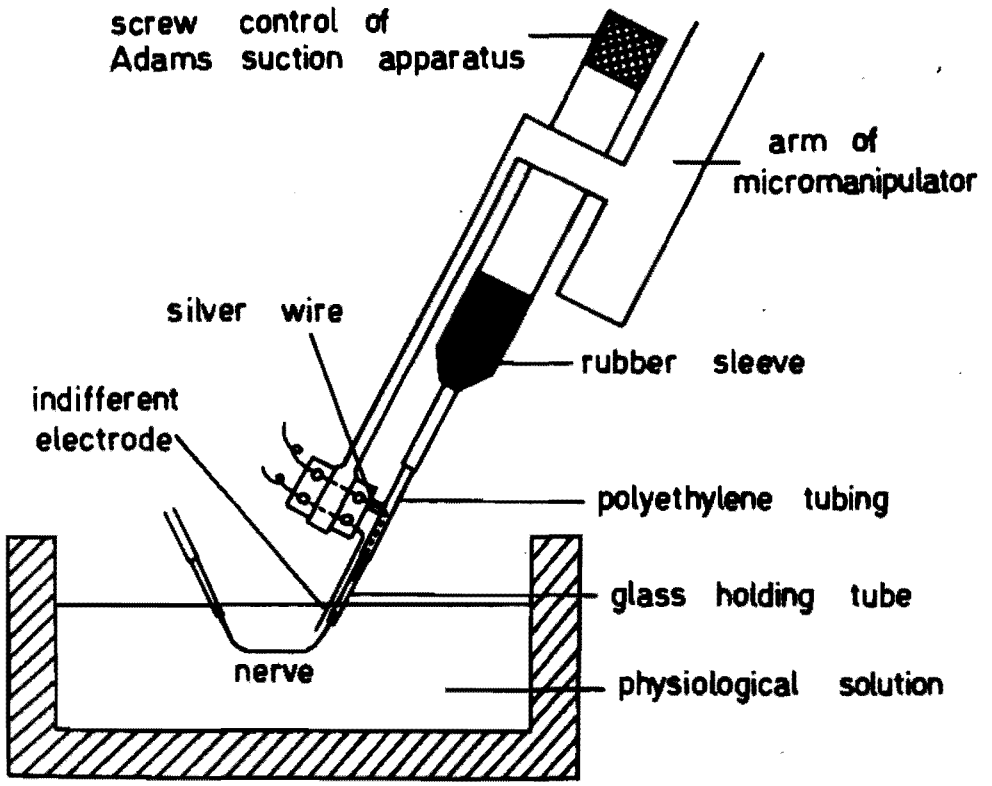

Figure 45: Suction-electrode assembly made in this work.

A P P E N D I X III

SUCTION-ELECTRODE ASSEMBLY

The assembly constructed in this work is shown in Figure 45. A Clay-Adams suction device, which essentially consists of an airtight gasket with a screw control, allowed adjustment of the hydraulic system. This suction device was connected to the electrode holding tube with polyethylene tubing. The electrode holding tubes were made from melting-point glass tubing with external diameter 2 mm. The end of each tube was drawn out to a fine tip, which was then fire-polished; tips with a range of diameters were made and they were calibrated using a series of B & S standard gauge wires (Nos. 20-36). Silver wire, inserted through the polyethylene tubing, served as an active electrode and a second electrode made of platinum foil was attached to the outside of the tube and served as an indifferent lead. The whole suction electrode assembly was clamped to a micromanipulator.

APPENDIX IV

PHYSIOLOGICAL SOLUTIONS FOR ELECTROPHYSIOLOGICAL EXPERIMENTS USING NERVES OF SCUTUS

Solution 1 (Normal solution) - based on cation concentrations found in the blood of animals equilibrated with normal sea water.

Salt	g/litre
NaCl	25.6596
KCl	0.8201
$CaCl_2 \cdot 6H_2O$	2.5195
$MgCl_2 \cdot 6H_2O$	10.1665
$Na_2SO_4 \cdot 10H_2O$	5.7998

Solution 2 ("75%")

Solution with cations in approximately the same concentration as in the blood of animals kept in 75% sea water for 24 hours.

Solution 3 ("environmental dilution")

Solution with cation concentrations approximately the same as the minimum blood concentration of animals subjected to changing sea water concentrations simulating conditions at the estuary.

Ion	Solution 1 mM/l.	Solution 2		Solution 3	
		mM/l.	% of soln 1.	mM/l.	% of soln 1.
Na^+	475	356	75	437.5	92.1
K^+	11.0	8.47	77	10.05	91.3
Ca^{2+}	11.5	8.97	78	10.10	87.8
Mg^{2+}	50.0	40.00	80	46.67	93.3
Cl^-	573	426.40	74.3	525.10	91.5
$SO4^{2-}$	18	18.00	100	18.00	100.0
pH	6.4	6.3		6.25	

Most marine invertebrates maintain their blood 0.5-1 pH unit more acid than sea water which has a pH of 8.0-8.1 (Prosser and Brown, 1961). The pH of a sample of blood from Scutus was found to be 7.1. Prosser and Brown have drawn attention to the fact that, for the measurement of pH, body fluids are drained in air and the loss of carbon dioxide might cause the recorded pH to be a little higher than that inside the animal. Hence the pH 6.4 of the physiological solution prepared from sodium, potassium, calcium and magnesium chlorides and sodium sulphate would probably not be much below that of blood inside Scutus. In invertebrates, except those with extensive calcium containing shells, most of the buffering of the hydrogen ions produced during respiration by the reaction

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \quad ,$$

is thought to reside in the blood proteins (Prosser and

Brown, 1961). As was found in Section 2, the protein concentration in the blood of Scutus is low and therefore the blood would not be expected to have a large buffering capacity, and pH's below 7 might be found in an animal.